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Sir:

Transmitted herewith for filing is a Continuation-in-Part application of prior Serial
No. PCT/US96/14659 filed September 13, 1996; and a Continuation-in-Part of U.S. Serial
Number 08/667,003 filed June 20, 1996; and a Continuation-in-Part of U.S. Serial Number
08/616,371 filed March 15, 1996 and 60/003,801 filed September 15, 1995.

The teachings of the above applications are incorporated herein by reference in their
entirety.

Inventor(s): Jonathan S. Stamler and Andrew J. Gow

Title: MODIFIED HEMOGLOBINS, INCLUDING NITROSYLHEMOGLOBINS, AND USES THEREFOR

- ☒ Specification, Claims, Abstract of the Disclosure
- ☒ 28 sheets of ~~formal~~ informal drawings. (Figs. 1A-1D, 2A, 2B, 3A, 3B, 4A-4D, 5, 6A-6F,
7A-7C, 8, 9A-9E, 10, 11, 12, 13, 14A, 14B, 15A, 15B, 16, 17, 18A, 18B and 19)
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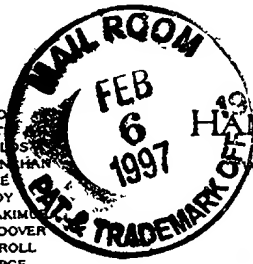
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Respectfully submitted,
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Dated: February 6, 1997



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Dated: February 6, 1997

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PATENT APPLICATION
Docket No.: DUK96-03pA3

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MODIFIED HEMOGLOBINS, INCLUDING NITROSYLHEMOGLOBINS,
AND USES THEREFOR

RELATED APPLICATIONS

This application is a continuation-in-part of
5 PCT/US96/14659 filed on September 13, 1996, and a
continuation-in-part of U.S. Serial Number 08/667,003 filed
on June 20, 1996, and a continuation-in-part of U.S. Serial
Number 08/616,371 filed on March 15, 1996. PCT/US96/14659
is a continuation of 08/667,003 filed on June 20, 1996 and
10 a continuation-in-part of 08/616,371 filed on March 15,
1996 and 60/003,801 filed on September 15, 1995. U.S.
Serial Number 08/667,003 is a continuation-in-part of U.S.
Serial Number 08/616,371, and a continuation-in-part of
U.S. Serial Number 60/003,801 filed September 15, 1995.
15 U.S. Serial Number 08/616,317 is a continuation-in-part of
U.S. Serial Number 60/003,801. The teachings of all of the
above applications are each incorporated herein by
reference in their entirety.

BACKGROUND OF THE INVENTION

20 Interactions of hemoglobin (Hb) with small diffusible
ligands, such as O₂, CO₂ and NO, are known to occur at its
metal centers and amino termini. The O₂/CO₂ delivery
functionalities, which arise in the lung and systemic
microvasculature, are allosterically controlled. Such
25 responsiveness to the environment is not known to apply in
the case of NO. Specifically, it has been thought
previously that Hb(Fe) is involved in limiting NO's sphere
of action (Lancaster, J.R., *Proc. Natl. Acad. Sci. USA*
91:8137-8141 (1994); Wood, J. and Garthwaite, J., *J.*
30 *Neuropharmacol.* 33:1235-1244 (1994)), but that NO does not

modify the functional properties of Hb to any physiologically significant degree. Kinetic modeling based on this assumption, however, predicts that the vast majority of free NO in the vasculature should be scavenged
5 by Hb (Lancaster 1994). Accordingly, the steady-state level of NO may fall below the K_m for target enzymes such as guanylate cyclase (Lancaster 1994), if not in the unperturbed organism, then with oxidant stress such as that found in atherosclerosis. These considerations raise the
10 fundamental question of how NO exerts its biological activity.

One answer to this paradox may be found in the propensity of nitric oxide to form S-nitrosothiols (RSNOs) (Gaston, B. et al., *Proc. Natl. Acad. Sci. USA*, 90:10957-
15 10961 (1993)), which retain NO-like vasorelaxant activity (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA*, 89:444-448 (1992)), but which are not subject to the diffusional constraints imposed by the high concentration of Hb in the blood. In particular, the NO group of RSNOs possesses
20 nitrosonium (NO^+) character that distinguishes it from NO itself. It is increasingly appreciated that RSNOs have the capacity to elicit certain functions that NO is incapable of (DeGroot, M.A. et al., *Proc. Natl. Acad. Sci. USA*, 92:6399-6403 (1995); Stamler, J.S., *Cell*, 78:931-936
25 (1994)). Moreover, consideration has been given to the possibility that -SNO groups in proteins may serve a signaling function, perhaps analogous to phosphorylation (Stamler, J.S. et al., *Proc. Natl. Acad. Sci. USA*, 89:444-448 (1992); Stamler, J.S. *Cell*, 78:931-926 (1994)).
30 Although S-nitrosylation of proteins can regulate protein function (Stamler, J.S. et al., *Proc. Natl. Acad. Sci. USA*, 89:444-448 (1992); Stamler, J.S., *Cell*, 78:931-936 (1994)), intracellular S-nitrosothiols -- the *sine qua non* of a regulatory posttranslational modification -- has heretofore
35 not been demonstrated.

Hemoglobin is a tetramer composed of two alpha and two beta subunits. In human Hb, each subunit contains one heme, while the beta (β) subunits also contain highly reactive SH groups (cys β 93) (Olson, J.S., *Methods in*
5 *Enzymology* 76:631-651 (1981); Antonini, E. & Brunori, M. In *Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York, pp. 29-31 (1971)). These cysteine residues are highly conserved among species although their function has remained elusive.

10 NO (nitric oxide) is a biological "messenger molecule" which decreases blood pressure and inhibits platelet function, among other functions. NO freely diffuses from endothelium to vascular smooth muscle and platelet and across neuronal synapses to evoke biological responses.
15 Under some conditions, reactions of NO with other components present in cells and in serum can generate toxic intermediates and products at local concentrations in tissues which are effective at inhibiting the growth of infectious organisms. Thus, it can be seen that a method
20 of administering an effective concentration of NO or biologically active forms thereof would be beneficial in certain medical disorders.

Platelet activation is an essential component of blood coagulation and thrombotic diathesis. Activation of
25 platelets is also seen in hematologic disorders such as sickle cell disease, in which local thrombosis is thought to be central to the painful crisis. Inhibition of platelet aggregation is therefore an important therapeutic goal in heart attacks, stroke, peripheral vascular disease
30 and shock (disseminated intravascular coagulation). Researchers have attempted to give artificial hemoglobins to enhance oxygen delivery in all of the above disease states. However, as recently pointed out by Olsen and coworkers, administration of underivatized hemoglobin leads
35 to platelet activation at sites of vascular injury (Olsen

S.B. et al., *Circulation* 93:327-332 (1996)). This major problem has led experts to conclude that cell-free underivatized hemoglobins may pose a significant risk in the patient with vascular disease or a clotting disorder (Marcus, A.J. and J.B. Broekman, *Circulation* 93:208-209 (1996)). New methods of providing for an oxygen carrier and/or a method of inhibiting platelet activation would be of benefit to patients with vascular disease or who are otherwise at risk for thrombosis.

10 SUMMARY OF THE INVENTION

The invention relates to methods of forming SNO-Hb by reaction of Hb with S-nitrosothiol in procedures which avoid oxidation of the heme. The invention also includes methods of producing nitrosated (including nitrosylated at thiols or metals) and nitrated derivatives of hemoglobins in which the heme Fe may or may not be oxidized, depending on the steps of the method. The invention also relates to a method of therapy for a condition in which it is desired to oxygenate, to scavenge free radicals, or to release NO⁺ groups to tissues. SNO-Hb in its various forms and combinations thereof (oxy, deoxy, met; specifically S-nitrosylated, or nitrosated or nitrated to various extents) can be administered to an animal or human in these methods. Thiols and/or NO donating agents can also be administered to enhance the transfer of NO⁺ groups. Examples of conditions to be treated by nitrosated or nitrated forms of hemoglobin include ischemic injury, hypertension, angina, reperfusion injury and inflammation, and diseases characterized by thrombosis.

30 BRIEF DESCRIPTIONS OF THE DRAWINGS

Figures 1A-1D are spectrographs of different forms of Hb as described in Example 1.

Figure 2A is a graph showing formation, with time, of SNO-Hb by S-nitrosylation.

Figure 2B is a graph showing the decomposition, with time, of oxy and deoxy forms of SNO-Hb.

- 5 Figure 3A is a graph showing the loading of red blood cells with S-nitrosocysteine, over time. The inset is a series of spectrographs of forms of Hb as described in Example 3.

- 10 Figure 3B is a series of tracings recording isometric tone of a rabbit aortic ring following treatment of the aortic ring with various agents as described in Example 3.

Figure 4A is a graph of change in tension of a rabbit aortic ring versus concentration of the Hb used in the experiment.

- 15 Figure 4B is a graph of change in tension of a rabbit aortic ring versus concentration of the Hb used in the experiment, where glutathione was also added to test the effect as compared to Figure 4A.

- 20 Figure 4C is a graph of the ratio of S-nitrosogluthathione formed/starting SNO-Hb concentration versus time, showing rates of NO group transfer from oxy and met forms of Hb to glutathione.

Figure 4D is a graph of S-nitrosothiols exported from loaded red blood cells over time.

- 25 Figure 5 is a graph showing the mean arterial blood pressure in rats after they received various doses of oxyHb (\blacktriangle), SNO-oxyHb (\blacksquare), or SNO-metHb (\bullet).

- 30 Figures 6A-6F are a series of tracings recording blood pressure (Figures 6A and 6B), coronary artery diameter (Figures 6C and 6D) and coronary artery flow (Figures 6E and 6F), after administration of S-nitrosohemoglobin to anesthetized dogs.

- 35 Figure 7A is a graph illustrating the effect of unmodified HbA₀ on platelet aggregation. The maximal extent of aggregation of platelets is plotted against the

concentration of HbA (10 nM to 100 μ M) preincubated with platelets. Experiments were performed as in Example 9. Vertical bars plotted with each data point indicate the standard deviation.

5 Figure 7B is a graph illustrating the effect of S-nitroso(oxy)hemoglobin on platelet aggregation. The normalized maximal extent of aggregation of platelets is plotted against the concentration of HbA (10 nM to 100 μ M) preincubated with platelets.

10 Figure 7C is a graph illustrating the antiaggregation effects on platelets by S-nitroso(met)hemoglobin.

Figure 8 is a bar graph showing the amount of cGMP (guanosine 3',5'-cyclic phosphoric acid), assayed as in Example 10, for 1, 10 and 100 μ M concentrations of native
15 Hb, SNO-oxyHb or SNO-metHb interacting with 10^8 platelets.

Figure 9A is a graph which shows the spectra (absorbance versus wavelength in nanometers) of HbA₀ treated as described in Example 11. The shift in the wavelength of maximum absorbance of spectrum B relative to
20 spectrum A illustrates the extent of addition of NO groups to HbA₀.

Figure 9B is a graph which shows the spectra of Hb treated with 100-fold excess S-nitrosoglutathione as described in Example 11.

25 Figure 9C is a graph which shows the spectra of HbA₀ treated with excess S-nitrosocysteine as described in Example 11.

Figure 9D is a graph which shows the spectra of rat Hb treated with 100-fold excess S-nitrosocysteine. Spectrum A
30 shows nitrosated Hb not further treated with dithionite; spectrum B shows nitrosated Hb further treated with dithionite.

Figure 9E is a graph illustrating the increase in nitrosated Hb product with time by reacting HbA₀ with
35 either 100x excess S-nitrosocysteine (top curve) or 10x

excess S-nitrosocysteine (middle curve). HbA₀ was preincubated with 100 μ M inositol hexaphosphate before reacting with 10x excess S-nitrosocysteine (bottom curve; triangle points). (See Example 11.)

5 Figure 10 is a graph illustrating the percent change, with time, in blood flow measured in caudatoputamen nucleus of rats after injection of the rats with: O, 100 nmol/kg SNO-Hb; ●, 1000 nmol/kg SNO-Hb; or ■, 1000 nmol/kg underivatized Hb (see Example 12).

10 Figure 11 is a graph illustrating the percent change in tension of a ring of aorta from rabbit, plotted as a function of the log of the molar concentration of hemoglobin tested (see Example 13). ●, Hb treated with S-nitrosocysteine at a ratio of 1:1 CYSNO/Hb; O, Hb treated
15 with CYSNO at a ratio of 10:1 CYSNO/Hb; ◆, Hb treated with CYSNO at a ratio of 100:1.

Figure 12 is a graph of the absorbance versus the wavelength of light (nm), for aqueous solutions of 17 μ M deoxyhemoglobin, 1 μ M NO, and varying amounts of dissolved
20 oxygen added by sequential injections of room air. The absorbance of the initial solution (no added air) is shown by the curve with the highest peak at approximately 430 nm. Sequential additions of 50 μ l of air shift the curve leftwards on the graph. See Example 14.

25 Figure 13 is a graph showing the yield of SNO-Hb as micromolar concentration (left axis, diamonds) and as % of NO added (right axis, squares), plotted against the heme:NO ratio, when nitrosyl-deoxyHb made at various ratios of heme:NO was exposed to oxygen. See Example 15.

30 Figure 14A is a graph showing difference spectra (each a spectrum of the NO and Hb mixture minus spectrum of the starting deoxyHb), for 17 μ M hemoglobin and NO mixtures, for the concentrations of NO shown. See Example 16.

Figure 14B is a graph showing the peak wavelength of the difference spectra plotted against the concentration of nitric oxide added to the solution as in Figure 14B.

Figure 15A is a graph showing difference spectra (deoxyhemoglobin and air mixtures minus initial deoxyhemoglobin spectrum), for successive additions of air.

Figure 15B is a graph showing difference spectra ($20 \mu\text{M}$ deoxyhemoglobin and $1 \mu\text{M}$ NO mixture, with successive additions of air, minus initial deoxyhemoglobin spectrum).

See Example 17.

Figure 16 is a graph showing two difference spectra (A_{418} of hemoglobin and NO solution at heme:NO 20:1 minus initial deoxyhemoglobin A_{418}) for the mutant $\beta 93\text{Ala}$ Hb and wild type $\beta 93\text{Cys}$ Hb. See Example 18.

Figure 17 is a graph showing the yield of SNO-Hb as micromolar concentration (left axis, diamonds) and as % of NO added (right axis, squares), plotted against the heme:NO ratio, when nitrosyl-deoxyHb made at various ratios of heme:NO was exposed to oxygen. See Example 19.

Figure 18A is a graph showing the percentage content of oxidized hemoglobin (metHb) for different concentrations of Hb (symbols below) to which NO was added to reach varying final concentrations (horizontal axis).

◆ represents $1.26 \mu\text{M}$ hemoglobin, ■ represents $5.6 \mu\text{M}$ hemoglobin, ▲ represents $7.0 \mu\text{M}$ hemoglobin, X represents $10.3 \mu\text{M}$ hemoglobin, ✕ represents $13.3 \mu\text{M}$ hemoglobin, and ● represents $18.3 \mu\text{M}$ hemoglobin. See Example 20.

Figure 18B is a graph showing the yield of oxidized hemoglobin (μM) plotted against the final concentration of NO added to solutions of Hb at the concentrations indicated by the symbols as for Figure 18A.

Figure 19 is a graph showing the concentration of oxidized Hb (metHb) plotted against the NO concentration, in experiments performed as described in Example 21 in 10

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mM (◆), 100 mM (Δ), or 1 M (✱) sodium phosphate buffer, pH 7.4.

DETAILED DESCRIPTION OF THE INVENTION

Roles for Hemoglobin in Physiology

5 The increase in SNO-Hb content of red cells across the pulmonary circuit (right ventricular inport-left ventricle) suggests that the Hb molecule is S-nitrosylated in the lung. Selective transfer of the NO group from endogenous RSNOs found in lung (Gaston, et al. (1993) and blood
10 (Scharfstein, J.S. et al., *J. Clin. Invest.* 94:1432-1439 (1995)) to SH groups of Hb, substantiate these findings. The corresponding decline in Hb(FeII)NO levels across the pulmonary bed reveals a role for the lung either in the elimination of NO or in its intramolecular transfer from
15 heme to cysβ93. Taken in aggregate, these data extend the list of function-regulating interactions of Hb with small molecules within the respiratory system, previously known to include the elimination of CO and CO₂ and uptake of O₂. Since oxygenation of Hb leads to structural changes that
20 increase the NO-related reactivity of cysβ93, O₂ may be regarded as an allosteric effector of Hb S-nitrosylation. This is a newly discovered allosteric function for the protein.

 The arterial-venous difference in SNO-Hb concentration
25 suggests that the protein acts as an NO group donor in the systemic circulation. There is good indication that SNO-Hb functions in regulation of vasomotor tone. In the microcirculation, where control of blood pressure is achieved, erythrocytes come in intimate contact with
30 endothelial surfaces. Under these conditions, Hb can contract the vasculature by sharply decreasing the steady state level of free NO (Lancaster, J.R., (1994). This is believed to contribute to the increases in blood pressure

that occur with infusion of cell-free Hbs (Vogel, W.M., et al., *Am. J. Physiol.*, 251:H413-H420 (1986); Olsen, S.B., et al., *Circulation* 93:329-332 (1996)). The transient nature of such hypertensive responses, however, is consistent with the subsequent formation of SNO-Hb which counteracts this effect, evidenced by its lowering of blood pressure at naturally occurring concentrations. Thus, the capacity of the erythrocyte to support the synthesis and metabolism of SNO-Hb may well be important for normal blood flow.

10 Mammals must have adopted unique molecular mechanisms to ensure adequate NO delivery in the microcirculation. Results herein suggest that Hb may have evolved both electronic and conformational switching mechanisms to achieve NO homeostasis. Specifically, NO scavenging by the metal center(s) of SNO-Hb(FeII)O₂ would be sensed through its conversion to met(FeIII) (Figure 1B). This electronic switch would effect decomposition of SNO-Hb with NO group release (Figures 3A, 3B, 4A). In this manner, the NO-related activity of SNO-Hb would be partly determined by the amount of NO scavenged. Changes in O₂ tension might also function to regulate NO delivery, as it is observed herein that NO release is facilitated by deoxygenation. This allosteric effect may promote the efficient utilization of O₂ as NO controls mitochondrial respiration (Shen, W., et al., *Circulation* 92:3505-3512 (1995)). It is also possible that NO group release serves to regulate capillary blood flow.

S-nitrosothiol groups in proteins have been implicated in NO metabolism and in regulation of cellular functions (Stamler, J.S., et al., *Proc. Natl. Acad. Sci USA*, 89:444-448 (1992); Stamler, J.S., *Cell*, 78:931-936 (1994)). The identification of SNO-Hb in erythrocytes is the first demonstration of an intracellular S-nitrosoprotein and gives further credence to the role of such proteins in cellular regulation. The question arises as to how SNO-Hb

relaxes blood vessels when any free NO released would be scavenged instantaneously by Hb itself (Lancaster, J.R., (1994)). Noteworthy in this regard are studies showing that RSNO activity involves nitrosyl (NO⁺) transfer to

5 thiol acceptors (Scharfstein, J.S., et al., (1994); Arnette, D.R. and Stamler, J.S., *Arch. Biochem. Biophys.* 318:279-285 (1995); Stamler, J.S., et al., *Proc. Natl. Acad. Sci USA*, 89:7674-7677 (1992)), which serve to protect the NO-related activity from inactivation at metal centers.

10 Findings presented herein indicate that S-nitrosothiol/thiol exchange with glutathione (forming GSNO) occurs within erythrocytes, and is influenced by the oxidation state of heme and its occupation by ligand. Certain activities of GSNO in bacteria require transport of

15 intact dipeptide (i.e., S-nitrosocysteinylglycine) across the cell membrane (DeGroote, M.A., et al., *Proc. Natl. Acad. Sci. USA* 92:6399-6403 (1995)). The data presented here show that S-nitrosothiol transport happens also in eukaryotic cells. GSNO, or related thiol carriers exported

20 by erythrocytes (Kondo, T., et al., *Methods in Enzymology*, Packer, L., ed., Academic Press, 252:72-83 (1995)), might also initiate signalling in or at the plasmalemma (Stamler, J.S., *Cell*, 78:931-936 (1994)), given reports of thiol-dependent activation of potassium channels by EDRF

25 (Bolotina, V.M., et al., *Nature*, 368:850-853 (1994)). Alternative possibilities also merit consideration. In particular, reports that Hb associates with red cell membranes via cys β 93 (Salhany, J.M. and Gaines, K.C., *Trends in Biochem. Sci.*, Jan, 13-15 (1981)) would place Hb

30 in a position to donate the NO group directly to contacting endothelial surfaces, perhaps via SNO/SH exchange. Cell surface interactions appear to be operative in signaling mediated by other S-nitrosoproteins (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA*, 89:444-448 (1992); Stamler,

35 J.S., *Cell*, 78:931-936 (1994)).

The highly conserved Cys β 93 residues in Hb influence the oxygen affinity and redox potential of the heme iron and its physiochemical properties (Garel, C., et al., *Biochem.* 123:513-519 (1982); Jocelyn, P.C., et al., *Biochemistry of the SH Group*, p.243, Academic Press, London; (1972); Craescu, C.T., *J. Biol. Chem.* 261:14710-14716 (1986); Mansouri, A., *Biochem. Biophys. Res. Commun.*, 89:441-447 (1979)). Nonetheless, their long sought-after physiological function has remained a mystery. The studies herein suggest new sensory and regulatory roles for Hb, in which Cys β 93 functions in transducing NO-related signals to the vessel wall. In particular, the physiological function of Cys β 93, which is invariant in all mammals and birds, is to deliver under allosteric control, NO-related biological activity that cannot be scavenged by heme. Thus, these data bring to light a dynamic circuit for the NO group in which intraerythrocytic Hb participates as both a sink and a donor, depending on its microenvironment. Such observations may provide answers to paradoxes that arise from conceptual frameworks based solely on diffusional spread and reaction of free NO (Lancaster, J.R., (1994); Wood and Garthwaite, *J. Neuropharmacology* 33:1235-1244 (1994)); and may have implications that extend to other thiol- and metal-containing (heme) proteins, such as nitric oxide synthase and guanylate cyclase.

The discoveries reported here may have direct therapeutic implications. Specifically, concerns over loss of NO-related activity due to inactivation by blood Hb (Lancaster, J.R., (1994)) are obviated by the presence of an RSNO subject to allosteric control. SNO-Hb is free of the adverse hypertensive properties of cell-free Hb preparations that result from NO scavenging at the metal centers. A cell-free Hb solution that mimics blood by containing SNO-Hb can be used as a blood substitute.

Further embodiments

The subject invention relates to a method of loading cells with a nitrosating agent as exemplified for red blood cells as in Figure 3A, but which can be accomplished in more general ways. Suitable conditions for pH and for the temperature of incubation are, for example, a range of pH 7-9, with pH 8 being preferred, and a temperature range of 25 to 37°C. For red blood cells, short incubation times of 1 to 3 minutes are preferred for limiting the formation of S-nitrosylated forms of Hb. However, intracellular concentrations of 1 mM nitrosating agent can be reached.

The nitrosating agent should be a good donor of NO⁺ and should be able to diffuse through the cell membrane of the target cell type. That is, it must be of low molecular weight, in contrast to S-nitroso proteins. Examples are S-nitroso-N-acetylcysteine, S-nitrosocysteinylglycine, S-nitrosocysteine, S-nitrosohomocysteine, organic nitrates and nitrites, metal nitrosyl complexes, S-nitro and S-nitroso compounds, thionitrites, diazeniumdiolates, and other related nitrosating agents as defined in Feelisch, M. and Stamler, J.S., "Donors of Nitrogen Oxides" chapter 7, pp. 71-115 *In Methods in Nitric Oxide Research* (Feelisch, M. and Stamler, J.S., eds.) John Wiley and Sons, Ltd., Chichester, U.K. (1996), the contents of which chapter are hereby incorporated by reference in their entirety.

Nitrosating agents have differential activities for different reactive groups on metal-containing proteins. A nitrosating agent can be chosen for minimal oxidation of the heme iron of Hb, and maximum activity in nitrosylating thiol groups such as found on cysteine. Assay methods are available for detection of nitrosation products, including S-nitrosothiols. See, for example Keefer, L.K., and Williams, D. L. H., "Detection of Nitric Oxide Via its Derived Nitrosation Products," chapter 35, pp. 509-519 *In Methods in Nitric Oxide Research* (Feelisch, M. and

Stamler, J.S., eds.) John Wiley and Sons, Ltd., Chichester, U.K., 1996; see also Stamler, J.S. and Feelisch, M., "Preparation and Detection of S-Nitrosothiols," chapter 36, pp. 521-539, *ibid.* Nitrite and nitrate products can be
5 assayed by methods described, for instance, in Schmidt, H.H.H.W. and Kelm, M., "Determination of Nitrite and Nitrate by the Griess Reaction," chapter 33, pp. 491-497, *ibid.*, and in Leone, A.M. and Kelm, M., "Capillary Electrophoretic and Liquid Chromatographic Analysis of
10 Nitrite and Nitrate," chapter 34, pp. 499-507, *ibid.*

Such low molecular weight nitrosating agents can be used in red blood cells to deliver NO-related activity to tissues. Treatment of red blood cells with nitrosating agent further serves to increase the O₂ delivery capacity
15 of red blood cells. Such treatment of red blood cells also allows for the scavenging of oxygen free radicals throughout the circulation. Therefore, it is possible to load red blood cells with S-nitrosothiol, for example, by a process outside a patient's body after removal of whole
20 blood (as a minimal method of isolating the red blood cells) and then to reintroduce the red blood cells into the same patient, thereby allowing the treatment of a number of types of diseases and medical disorders, such as those which are characterized by abnormal O₂ metabolism of
25 tissues, oxygen-related toxicity, abnormal vascular tone, abnormal red blood cell adhesion, or abnormal O₂ delivery by red blood cells. Such diseases can include, but are not limited to, ischemic injury, hypertension, shock, angina, stroke, reperfusion injury, acute lung injury, sickle cell
30 anemia, schistosomiasis and malaria. The use of such "loaded" red blood cells also extends to blood substitute therapy and the preservation of living organs, as organs for transplantation, for example. In some cases, it may be appropriate to treat a patient with loaded red blood cells
35 originating from a different person.

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A particular illustration of the mechanism of the treatment method is presented here by considering sickle cell anemia. Sick cell patients suffer from frequent vascular occlusive crises which manifest in clinical syndromes such as the acute chest syndrome and hepatic dysfunction. Both endothelial cell dysfunction, resulting in a clotting diathesis as well as dysfunction intrinsic to the red blood cell, are central to disease pathogenesis. At the molecular level, the increased expression of vascular adhesion molecules such as VCAM promote the adhesion of sickled red blood cells containing abnormal hemoglobin. It follows that decreasing cytokine expression on endothelial cells, promoting endothelial function and attenuating red cell sickling, are key therapeutic objectives. However, currently used therapies have been generally unsuccessful.

In this novel method for loading red blood cells with intracellular NO-donor S-nitrosothiols, the effect is to increase oxygen affinity -- which in and of itself should attenuate red blood cell sickling -- and to endow the red blood cell with vasodilator and antiplatelet activity, which should reverse the vasoocclusive crisis. Moreover, nitric oxide should attenuate the expression of adhesion molecules on endothelial cell surfaces, thus restoring endothelial function.

Herein is described a novel therapeutic approach to the treatment of sickle cell disease which involves loading of red blood cells with S-nitrosothiols or other nitrosating agents. Two examples of therapeutic approaches are given. In the first, the patient's own red blood cells are S-nitrosated extracorporeally (yielding "loaded" red blood cells) and then given to the patient. The second approach is to directly administer to a patient an agent such as S-nitrosocysteine, which is permeable to red blood cells.

For some diseases or disorders, the administration of NO-loaded red blood cells is especially desirable. Upon a change from the oxygenated to the deoxygenated state, or upon a change in the oxidation state of the heme Fe from the reduced state (FeII) to the oxidized (FeIII) state, NO is released from the thiol groups of hemoglobin, and is rapidly transferred to glutathione to form S-nitrosoglutathione. Red blood cells are known to have a high concentration of glutathione. S-nitrosoglutathione efficiently delivers NO to tissues.

In another aspect, the invention is a method for the treatment of infection by administering to an infected mammal an agent which causes S-nitrosation of thiol groups within the cells which are the target of such agent. For example, an S-nitrosothiol to which lymphocytes are highly permeable can be administered to a patient infected with HIV. Such treatment for HIV can also be used excorporeally, to blood isolated from the patient. In another application, the infection is bacterial, and the S-nitrosothiol to be used as an anti-bacterial agent is one to which the target bacterial cells are highly permeable, as compared to the permeability properties of the host cells. (See, for example De Groote, M.A., et al., *Proc. Natl. Acad. Sci. USA* 92:6399-6403 (1995).) Alternatively, nitrosothiols can be used to treat *Plasmodium falciparum* within red blood cells.

Another embodiment of the invention is a method for specifically modifying a protein containing one or more metal atoms so that the protein becomes S-nitrosylated at one or more thiol groups without modifying the metal, as by changing the oxidation state or causing the metal atoms to bind NO. This can be accomplished by the use of a reagent which possesses NO⁺ character, such as a nitrosothiol (See, for instance, Example 4A.), which reacts specifically with thiol groups of a protein in which metal is bound.

An S-nitrosation method has been devised which does not affect the heme of hemoglobin. SNO-Hb (SNO-Hb(FeII)O₂) can be synthesized from Hb(FeII)O₂ with up to 2 SNO groups per tetramer without oxidation of the heme Fe from FeII to FeIII. In contrast, when Hb(FeII)O₂ is incubated with excess nitric oxide or nitrite, methemoglobin (HbFe[III]) forms rapidly (Example 1B) and to a significant extent. When Hb[FeII] is incubated with nitric oxide, NO binds rapidly to the heme, forming Hb(FeII)NO to a significant extent (Example 1A).

Although rates of formation of SNO-Hb(FeII)O₂ from Hb(FeII)O₂ are more rapid (see Example 2A), the corresponding SNO-deoxyHb form can also be made by incubation of S-nitrosoglutathione or S-nitrosocysteine, for example, with Hb(FeII), yielding SNO-Hb(FeII), as in Example 1C.

The effects of the various forms of Hb on vasodilation -- constriction, dilation or a neutral effect -- depend on three factors: whether 1) the Fe of the heme is oxidized, 2) O₂ is bound at the heme (that is, the oxygenation state, dictated by the conformation of the protein as R state or T state), and 3) thiol is present in sufficient concentration to facilitate the transfer of NO⁺.

The importance of the first factor is shown in Figure 4A. Hb(FeII)O₂ and SNO-Hb[FeII]O₂ act as vasoconstrictors, but SNO-Hb[FeIII] (metHb form, where FeII has been oxidized to FeIII) acts as a vasodilator. Figure 4A shows that SNO-Hb[FeII]O₂ with oxygen bound at the heme, and with a ratio of SNO/Hb=2, acts as a powerful vasoconstrictor.

SNO-Hb(FeII) is also a vasodilator. Figure 2B illustrates the second factor in demonstrating that rates of RSNO decomposition and transfer are much faster for SNO-Hb in the deoxy state than for SNO-Hb in the oxy state.

It can be seen how the NO⁺-donating properties of SNO-Hb depend on oxygen concentrations. SNO-Hb releases oxygen

at sites of low oxygen concentration or under oxidizing conditions. SNO-Hb releases its NO group(s) to cause vasodilation either due to 1) oxidation of the heme Fe to FeIII or 2) loss of the O₂ from the heme by deoxygenation.

5 It is shown in Figure 2B that NO is transferred off SNO-Hb best in the deoxy state. In ischemia, SNO-Hb deoxygenates, rapidly followed by the loss of NO. It can be seen from the data that SNO-metHb having a ratio of 1 SNO/SNO-metHb is a more powerful vasodilator than SNO-oxyHb having a
10 ratio of 2 SNO/SNO-oxyHb. It should be noted that S-nitrosation of Hb induces the R state (oxy conformation). Thus, it follows that 1 SNO-oxyHb molecule having a ratio of 1 SNO/SNO-oxyHb is less potent than 10 molecules of SNO-oxyHb having a ratio of 0.1 SNO/SNO-oxyHb.

15 The third factor is illustrated by the results shown in Figure 4B. These results demonstrate potentiation by thiol of the vasodilator effect of SNO-Hb(FeII)O₂ and SNO-Hb(FeIII). Transfer of NO⁺ from SNO-Hb to low molecular weight nitrosothiols is more efficient when Hb is in the
20 deoxy state compared to the oxy state (Figure 2B) or in the met state compared to the oxy state (Figure 4C).

NO is released or transferred as NO⁺ (nitrosyl cation) from SNO-Hb. The SNO groups of SNO-Hb have NO⁺ character. Transfer of NO⁺ from SNO-Hb occurs most efficiently to
25 small thiols, such as glutathione, and is most efficient when the heme is oxidized (SNO-metHb) or the SNO-Hb is in the deoxy state.

One embodiment of the invention resulting from these findings is a method of therapy that enhances the transfer
30 of NO⁺ from SNO-Hb to small thiols, thereby delivering NO biological activity to tissues, by the coadministration of low molecular weight thiols, along with a form of SNO-Hb, to a mammal in need of the physiological effects of NO. To further increase the effect of NO release it is preferred
35 that the SNO- forms of metHb or deoxyHb (or an equivalent

conformation or spin state) be administered with the thiol (See Figure 2B, for example.) A mixture of SNO-metHb and SNO-oxyHb, and possibly also thiol, can also be used. The composition and proportion of these components depends on the disease state. For example, to achieve both enhanced O₂ delivery and NO delivery, SNO-oxyHb can be used. Where no further delivery of O₂ is desirable, as in acute respiratory distress syndrome, for example, the SNO- forms of metHb and deoxyHb are especially preferred.

10 Alternatively, the ratios of SNO/Hb can be regulated to control O₂ release.

The vessel ring bioassay data of Figure 4A agree well with the *in vivo* data of Figure 5. The results of the experiments described in Example 5 confirm that Hb(FeII)O₂ (oxyHb) causes an increase in blood pressure *in vivo*, as it did also *in vitro*. SNO-Hb(FeIII) (SNO-metHb) causes a decrease in blood pressure *in vivo* as well as *in vitro*. SNO-Hb(FeII)O₂ (SNO-oxyHb) has a negligible effect on blood pressure *in vivo* in contrast to the increase in tension seen in the corresponding vessel ring bioassay. For SNO-oxyHb the *in vivo* effect is neutral. This may be explained by the constrictive effect caused by NO becoming bound to the heme being compensated by the release of NO upon deoxygenation. Therefore, SNO-oxyHb can deliver O₂ with minimal effect on blood pressure.

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With knowledge of the results herein it is possible to synthesize Hb proteins with predicted NO-releasing properties, which will constrict, dilate, or have no effect on blood vessels. An additional option is the choice between making oxygenated or deoxygenated forms to administer for medical conditions in which O₂ delivery is desirable, or undesirable, respectively.

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It is possible to produce a variety of modified Hbs having specific desired properties of O₂ and NO delivery. For example, Hb in the R state or R-structure (oxyHb) can

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be converted to the T state or T-structure (deoxyHb) by a number of known methods. This can be done, for example, by reaction of Hb with inositol hexaphosphate. It is also known to those skilled in the art that Hb in the R state
5 can be made, for example, by treating Hb with carboxypeptidase. Similarly, it is known that metHb can be synthesized using ferricyanide or nitrite.

Producing Hb molecules which are locked in the T state allows the synthesis of RSNO-Hb which remains in a form
10 that is a biologically active donor of NO, rather than a carrier of NO. Hb which is locked in the R state can be used as a substrate for the synthesis of RSNO-Hb which carries a maximum amount of NO per molecule.

Another embodiment of the invention is a blood
15 substitute comprising one or more forms of Hb which have been specifically S-nitrosated to some extent at one or more thiol groups of the Hb, in order to regulate O₂ release and NO release. Conditions to be treated include those in which NO or O₂ delivery is desired, those in which
20 NO or O₂ utilization is desired, or those in which NO or O₂ is in excess. For example, in a medical condition which is characterized by the presence of an excess of oxygen free radicals and excess NO[•], both the heme of SNO-Hb and NO released by SNO-Hb serve to trap oxygen free radicals. The
25 heme Fe is oxidized in the process of scavenging oxygen free radicals and NO[•], and NO is released from the oxidized Hb by donation to a thiol, in the form of RSNO⁺, which is not toxic. Inflammation and reperfusion injury, for example, are characterized by excess NO production and an
30 excess of oxygen free radicals. Forms of Hb scavenge oxygen radicals and free NO, converting NO to forms that are not toxic.

A further embodiment of the invention is a method of therapy for a condition that would benefit from the
35 delivery of NO in a biologically active form or O₂ or both,

based on the administration of a blood substitute comprising a form of nitrosated Hb. For example, SNO-Hb is useful to treat myocardial infarction. SNO-Hb has the effect of donating NO, keeping blood vessels open. SNO-Hb deoxygenates at low oxygen tension, delivering oxygen and releasing NO at the same site, thereby causing vasodilation. (See Example 7 and Figures 6A-6F.) These effects can be augmented by also administering thiol, either simultaneously with SNO-Hb, or before or after. For the purpose of treating myocardial infarction, for example, a high concentration or dose of SNO-Hb that has a low ratio of SNO/SNO-Hb is appropriate. Alternatively, SNO-metHb can be used for this purpose.

In another aspect, the invention is a method of enhancing NO-donor therapy by coadministering SNO-Hb or other forms of nitrosated Hb together with a nitroso-vasodilator (nitroglycerin, for example) which would be otherwise consumed by the conversion of oxyHb to metHb in Hb which has not been S-nitrosated.

Platelet activation is manifested by a number of events and reactions which occur in response to adhesion of platelets to a nonplatelet surface such as subendothelium. Binding of agonists such as thrombin, epinephrine, or collagen sets in motion a chain of events which hydrolyzes membrane phospholipids, inhibits adenylate cyclase, mobilizes intracellular calcium, and phosphorylates critical intracellular proteins. Following activation, platelets secrete their granule contents into plasma, which then allow the linking of adjacent platelets into a hemostatic plug. (See pages 348-351 in *Harrison's Principles of Internal Medicine*, 12th edition, eds. J.D. Wilson et al., McGraw-Hill, Inc., New York, 1991).

A thrombus is a pathological clot of blood formed within the circulatory system. It may remain attached to its place of origin or become dislodged and move to a new

site within the circulatory system. Thromboembolism occurs when a dislodged thrombus or part of a thrombus partially or completely occludes a blood vessel and prevents oxygen transport to the affected tissues, ultimately resulting in tissue necrosis.

Sites where damage has occurred to the vascular surface are especially susceptible to the formation of thrombi. These sites include those on the interior surface of a blood vessel in which damage to the endothelium, narrowing or stenosis of the vessel, or atherosclerotic plaque accumulation has occurred.

NO is one of several endothelium-derived thromboregulators, which are defined as physiological substances that modulate the early phases of thrombus formation. In particular, NO reduces platelet adhesion, activation and recruitment on the endothelial cell surface, and achieves this, it is thought, by activating platelet guanylate cyclase, thereby increasing platelet intracellular CGMP (Stamler, J.S. et al, *Circ. Res.* 65:789-795 (1989)), and decreasing intraplatelet Ca^{2+} levels. NO and the prostacyclin prostaglandin (PG) I_2 act synergistically to inhibit and actively mediate platelet disaggregation from the collagen fibers of the subendothelial matrix. Unlike prostacyclin, NO also inhibits platelet adhesion. Furthermore, platelets synthesize NO, and the L-arginine-NO pathway acts as an intrinsic negative feedback mechanism to regulate platelet reactivity. NO is involved in leukocyte interactions with the vessel wall and can inhibit neutrophil aggregation. (See review article, Davies, M.G. et al., *British Journal of Surgery* 82:1598-1610, 1995.)

NO is antiathrogenic in a number of ways. (See, for example, Candipan, R.C. et al., *Arterioscler. Thromb. Vasc. Biol.* 16:44-50, 1996.) NO inhibits smooth muscle

proliferation and attenuates LDL (low density lipoprotein) oxidation and other oxidant-related processes.

Hemoglobin may promote atherosclerosis as well as thrombosis as a consequence of its NO-scavenging property.

- 5 This limitation of hemoglobin derives from its high affinity for nitric oxide. *In vitro*, NO is a potent inhibitor of platelet aggregation and adhesion to collagen fibrils, the endothelial cell matrix and monolayers (Radomski, M.W. et al., *Br. J. Pharmacol.* 92:181-187
10 (1987); Radomski, M.W. et al., *Lancet* 2:1057-1058 (1987); Radomski M.W. et al., *Biochem. Biophys. Res. Commun.* 148:1482-1489 (1987)). NO elevates CGMP levels in platelets, thereby decreasing the number of platelet-bound fibrinogen molecules and inhibiting intracellular Ca^{++} flux
15 and platelet secretion (Mellion, B.T. et al., *Blood* 57:946-955 (1981); Mendelson, M.E. et al., *J. Biol. Chem.* 165:19028-19034 (1990); Lieberman, E. et al., *Circ. Res.* 68:1722-1728 (1991)). Scavenging of nitric oxide by Hb prevents the molecule from inhibiting platelets. This
20 explanation has been given support by *in vivo* studies (Krejcy, K. et al., *Arterioscler. Thromb. Vasc. Biol.* 15:2063-2067 (1995)).

- The results shown in Figures 7A-7C (see Example 9) show that nitrosated hemoglobins, including SNO-Hb, can be
25 used in the treatment of acute blood clotting events that occur as a result of increased platelet deposition, activation and thrombus formation or consumption of platelets and coagulation proteins. Such complications are known to those of skill in the art, and include, but are
30 not limited to myocardial infarction, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis and unstable angina, and any additional complication which occurs either directly or indirectly as a result of the foregoing disorders.

SNO-Hb and other nitrosated hemoglobins can also be used prophylactically, for example, to prevent the incidence of thrombi in patients at risk for recurrent thrombosis, such as those patients with a personal history
5 or family history of thrombosis, with atherosclerotic vascular disease, with chronic congestive heart failure, with malignancy, or patients who are pregnant or who are immobilized following surgery.

NO is known to activate soluble guanylate cyclase,
10 which produces CGMP. CGMP mediates inhibition of platelet aggregation. Results in Example 10 demonstrate that this inhibition of platelet aggregation may be mediated not by CGMP alone, but by some other mechanism as well.

Certain compounds or conditions are known to cause a
15 shift in the allosteric equilibrium transition of Hb towards either of the two alternative quaternary structures of the tetramer, the T- or R-structures. (See, for example, pages 7-28 in Perutz, M., *Mechanisms of Cooperativity and Allosteric Regulation in Proteins*,
20 Cambridge University Press, Cambridge, U.K., 1990.) These are, for instance, the heterotropic ligands H^+ , CO_2 , 2,3-diphosphoglycerate (2,3-DPG) and Cl^- , the concentrations of which modulate oxygen affinity. The heterotropic ligands lower the oxygen affinity by forming additional hydrogen
25 bonds that specifically stabilize and constrain the T-structure. Other compounds affecting the allosteric equilibrium include inositol hexaphosphate (IHP) and the fibric acid derivatives such as bezafibrate and clofibrate. The fibric acid derivatives, antilipidemic drugs, have been
30 found to combine with deoxy-, but not with oxyhemoglobin. They stabilize the T-structure by combining with sites in the central cavity that are different from the DPG binding sites. Other allosteric effectors have been synthesized which are related to bezafibrate. A ligand that stabilizes
35 specifically the R-structure increases the oxygen affinity,

and a ligand that stabilizes the T-structure does the reverse. Other ligands may affect the spin state of the heme. For example, in deoxyhemoglobin and in methemoglobin the Fe is high-spin ferrous ($S=2$) and 5-coordinated; in
5 oxyhemoglobin and in cyan-metHb the Fe is low-spin ferrous ($S=0$) and 6-coordinated; when H_2O is the sixth ligand, methemoglobin is also high-spin. The inhibition of platelet aggregation by S-nitroso-methemoglobin seen in
10 Figure 7C is consistent with enhanced potency in the high spin conformation. Such substances which control the allosteric equilibrium or spin state of hemoglobin may be administered to a human or animal to promote the formation of, or to stabilize, a particular allosteric structure and/or spin state.

15 The dosage of Hb required to deliver NO for the purpose of platelet inhibition can be titrated to provide effective amounts of NO without causing drastic changes in blood pressure. If the goal of the therapy is to deliver oxygen, the Hb may be administered in a unit of blood to
20 avoid a drop in blood pressure. If the goal is to alleviate shock, very little Hb can be administered compared to the amount to be given for myocardial infarction. For shock, the more important goal is to deliver NO rather than to deliver oxygen. It may be
25 preferable to use continuous infusion or several infusions per day. Example 12 (see Figure 10) shows that the effects of $SNO-Hb(FeII)O_2$ on blood flow in rat brain last over 20 minutes; in other experiments an effect has been seen for up to an hour. There is a correlation between blood
30 pressure effects and platelet inhibition effects, but platelet inhibition occurs at a lower NO concentration than that which is required to produce blood pressure effects, and generally lasts longer.

Example 11 shows that S-nitrosothiols can be used to
35 add NO groups not only on the thiol groups of cysteine

residues in hemoglobin, but also on other reactive sites of the hemoglobin molecule. The products of the nitrosation reactions in Example 11 were hemoglobin molecules with more than 2 NO groups per Hb tetramer. The exact sites of the addition of NO have not been confirmed, but it is expected that NO addition occurs at thiol groups and various other nucleophilic sites within Hb, including metals. Reactive sites, after the thiol groups, are tyrosine residues and amines, and other nucleophilic centers.

10 Nitrosation reactions on other proteins have been investigated previously (Simon, D.I. et al., *Proc. Natl. Acad. Sci. USA* 93:4736-4741 (1996)). Methods of modifying proteins to produce nitrosoproteins are known in the art, and include, for example, exposing the protein to NaNO_2 in 15 0.5 M HCl (acidified NO_2^-) for 15 minutes at 37°C. An alternative method is to place a helium-deoxygenated solution of protein in 100 mM sodium phosphate, pH 7.4, inside dialysis tubing and expose the protein to NO gas bubbled into the dialysate for 15 minutes. (Stamler, J.S. 20 et al., *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992); see also Williams, D.L.H. *Nitrosation*, Cambridge University Press, New York (1988), which gives further methods of nitrosation).

By these methods, multiple NO-related modifications 25 ("NO groups" or "NO biological equivalents" resulting from nitrosations, nitrosylations or nitrations) can be made on Hb at nucleophilic sites, which may include thiols, nucleophilic oxygen atoms as may be found in alcohols, nucleophilic nitrogen atoms as may be found in amines, or 30 the heme iron. Agents facilitating nitrosations, nitrosylations or nitrations of Hb can be thought of as "NO or NO^+ donating agents." The products of such modifications may have such groups, for example, as -SNO, -SNO₂, -ONO, ONO₂, -CNO, -CNO₂, -NNO, -NNO₂, -FeNO, -CuNO,

-SCuNO, SFeNO and the different ionized forms and oxidation variants thereof. (See, regarding oxidation of hemoglobin by Cu^{++} , Winterbourne, C., *Biochemistry J.* 165:141-148 (1977)). The covalent attachment of the NO group to

5 sulfhydryl residues in proteins is defined as S-nitrosation; the covalent attachment of the NO group to a metal, such as Fe, can be called nitrosylation, yielding a metal-nitrosyl complex. General NO attachment to nucleophilic centers is referred to herein as nitrosation. Thus, the term
10 nitrosated hemoglobin as used herein includes SNO-Hb and Hb(FeII)NO as well as other forms of hemoglobin nitrosated at other sites in addition to thiols and metals. In addition, Hb can be nitrated. Hbs which have been nitrosated and/or nitrated at multiple different types of
15 nucleophilic sites (termed polynitrosated, that is, having NO equivalents added to other nucleophilic sites as well as to thiols; or polynitrated, respectively) will permit transnitrosation reactions and the release of NO and its biological equivalents in the circulatory system at
20 different rates and engendering different potencies.

These and other nitrosation and nitration reactions can cause oxidation of the heme Fe to some extent, under some conditions. However, some minor degree of oxidation is acceptable. The nitrosated Hb is still be useful as a
25 therapeutic agent if oxidized to a minor extent. For applications where the NO-delivering function, rather than the O_2 -delivering function of nitrosated Hb, is more desirable, extensive oxidation of the heme Fe is acceptable.

30 If it is desired to avoid oxidation of the heme Fe, it is possible to remove the heme, perform the necessary chemical reactions upon the protein to nitrosate to the extent desired, and replace the heme into the modified hemoglobin product. (See, for removing and replacing the
35 heme, Antonini, E. and Brunori, M., *Hemoglobin and*

Myoglobin in their Reactions with Ligands, Elsevier, New York, 1971.)

In addition to the nitrosating under conditions that do not oxidize the heme, such as brief exposure to low molecular weight RSNOs, as illustrated in Examples 1 and 2, alternative methods can be used to produce nitrosated hemoglobin in which the heme Fe is not oxidized. For instance, it is possible to produce by recombinant methods α and β globin chains, nitrosate them to the extent desired, then assemble the chains with heme to form a functional, nitrosated tetramer. (See, for example, European Patent Application EPO 700997, published March 13, 1996, filed May 10, 1990, "Production in bacteria and yeast of hemoglobin and analogues thereof.")

Another alternative method to nitrosate the α and β globin chains without producing a form of metHb as the end product, is to nitrosate the intact Hb molecule to the extent desired, thereby allowing the heme Fe to be oxidized, then reduce the heme Fe by treating the nitrosated Hb with either methemoglobin reductase or a cyanoborohydride such as sodium cyanoborohydride.

It has been generally thought that nitric oxide as NO gas in solution reacts with hemoglobin (Hb) in two major ways: 1) with the deoxyHb to form a stable nitrosyl (FeII) heme adduct; and 2) with oxyHb to form nitrate and metHb -- a reaction that inactivates NO. These two reactions contributed to the idea that Hb is a scavenger of NO. In both of these reactions, NO biological activity is lost. The results described herein demonstrate that, in fact, neither reaction occurs under physiological conditions. Rather, the products of the NO/Hb reaction are dictated by the ratio of NO to Hb, and by the conformation of Hb -- R(oxy) vs. T(deoxy).

At low ratios of NO to deoxyHb (e.g., 1:100 or less), the Hb molecule is in T-structure. Under this condition,

NO introduced as gas to a Hb solution binds to the α -hemes, as has been seen by EPR. Upon introduction of oxygen, with conversion to the R state, NO is transferred to a thiol of cysteine to yield S-nitrosohemoglobin with close to 100% efficiency. At ratios of NO/Hb of 1:25-1:50, the efficiency of formation of SNO-Hb is ~35% (decreasing with increasing NO/Hb ratio). The reaction appears to involve migration of NO from α heme to β heme and then to the β thiol. In going from the heme to a thiol, the heme or nitrosothiol needs to lose an electron by oxidation ($\text{NO} \rightarrow \text{NO}^+$ or $\text{RSNO} \cdot \rightarrow \text{RSNO}$). Oxygen serves as an electron acceptor in the system, driving the reaction thermodynamically, as well as causing a conformational change by its binding at the heme, which exposes the thiol groups. At higher ratios of NO to Hb (1:20-1:2), with the protein still in T-structure, we find that the protein liberates NO^- from the β hemes with production of metHb. This occurs in the absence of O_2 and provides another indication that the NO bound to β -hemes is unstable. Once O_2 is introduced, S-nitrosothiol (SNO) may form, but the relative yield is very low because of loss to NO^- . The yield of SNO-Hb approaches zero at NO/Hb ratios of 1:2, upon introduction of oxygen.

At the higher ratios of NO to Hb (i.e., >0.75-1), NO itself maintains the R-structure. Under this condition, the NO is more stable because of an unusual constraint on the molecule. Specifically, loss of NO from the β hemes promotes the T-structure, whereas formation of SNO-Hb selects for the R-structure. This is not a favored reaction. The consequence is that small amounts of S-nitrosohemoglobin are formed, but the yields are low (~5%). This does not exclude the possibility that the molecule has therapeutic value, but would give the appearance of a very stable compound with no biological effect.

The reaction of NO with oxyHb is also dependent on the ratios of NO to oxyHb. Under conditions of relatively high (non-physiological) ratios of NO to Hb, (NO/oxyHb >1:20), NO appears to destabilize the hydrogen bond between the O₂ and the proximal histidine (by competing for it) yielding some metHb. By changing the ionic composition of the solvent buffer (e.g., borate 0.2 M, pH 7.4), formation of metHb can be significantly reduced even with excess NO (NO/Hb = 3:1). On the other hand, metHb formation is facilitated in acetate buffer at pH 7.4; when the hydrogen bond between O₂ and the proximal histidine is broken, the O₂ seems to gain superoxide-like character. NO then reacts rapidly to form metHb and nitrate. Efficient metHb formation actually requires an excess of NO/oxyHb. In contrast, at lower ratios of NO/Hb (<1:20), it reacts with the small residual fraction (<1%) of deoxyHb, in turn producing S-nitroso-hemoglobin extremely efficiently. As the concentration of NO is increased, there is some reaction with oxyHb, but the products are nitrite and nitrate, not nitrate alone. The conclusion is that NO can be incubated in reaction mixtures of oxyHb without inactivating the O₂ binding functionality by converting it to nitrate.

Nitrosylhemoglobin can be used in an animal or human as a therapeutic NO donor for the prevention or treatment of diseases or medical disorders which can be alleviated by delivery of NO or its biologically active form to tissues affected by the disease or medical disorder. Like SNO-Hb, nitrosylhemoglobin can be administered as a blood substitute, because nitrosylhemoglobin can be converted to SNO-Hb under physiological conditions. NO is released from the thiol either by deoxygenation or by conversion to metHb.

Inhaled NO causes selective pulmonary vasodilation without influencing systemic responses. A previously-

formed rationale behind its use is that scavenging by Hb prevents adverse systemic effects. It is illustrated in Examples 14-21 that NO can be used to produce S-nitrosohemoglobin, which is a potent vasodilator and antiplatelet agent. Inhaled NO can be used to raise levels of endogenous S-nitrosohemoglobin. Similarly, treatment of red blood cells (RBCs) with NO can be used to form SNO-RBCs, or "loaded" red blood cells.

Compared to SNO-deoxyHb, which is a good NO donor, but which would release its NO very quickly, or SNO-oxyHb, which would release its NO more slowly, but has a propensity to form metHb over time, nitrosyl-deoxyhemoglobin stored in a form such that final ratio of NO:heme is less than about 1:100 or greater than about 0.75, is stable. Formation of metHb is prevented at these NO:heme ratios. For this reason nitrosyl-deoxyhemoglobin stored with such NO:heme ratios in a physiologically compatible buffer can be administered to an animal or human as an NO donor.

A blood substitute or therapeutic which can be used as an NO donor, and which is free of the vasoconstrictor effects of underviatized Hbs, can be made by obtaining a solution of oxyHb (including solutions stored in the form of oxyHb) and adding NO as dissolved gas, yielding SNO-oxyHb. Buffer conditions and NO:Hb ratios can be optimized, as illustrated in Example 21 and Figure 19, to yield S-nitrosothiol without significant production of oxidized Hb (metHb). For example, NO added to oxyhemoglobin in 10 mM phosphate buffer, pH 7.4, at a ratio of less than 1:30 NO:Hb resulted in formation of SNO-oxyHb with minimal formation of metHb. This ratio can be increased by varying the buffer conditions, for example by the use of 10 mM phosphate, 200 mM borate at pH 7.4. The buffer anions as well as the buffer concentration should be chosen carefully. For instance, acetate and chloride have

the opposite effect from borate, increasing the formation of metHb and nitrite at 200 mM, pH 7.4.

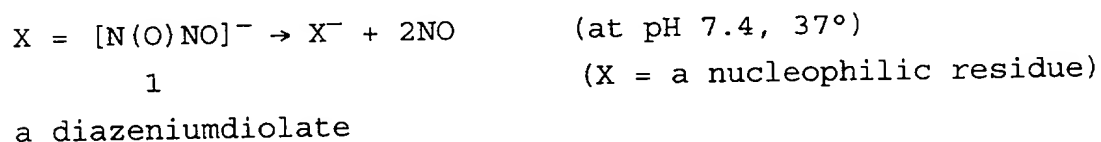
This may be explained by a competition between free NO and oxygen for a H-bond with the imidazole of the proximal
5 His residue. If low concentrations of NO are used, in low ionic strength buffer, e.g., 10 mM phosphate, metHb does not readily form. If the H-bond is weakened by increasing the ionic strength of the buffer, NO reacts more readily with oxyHb, yielding more metHb. Buffers with a low pK_a
10 relative to pH 7.4 tend to stabilize FeIII. Buffers having a pK_a at least about two pH units higher than the reaction condition are preferred.

A blood substitute can be made which acts as a donor of NO^- . NO can be added to a solution of deoxyHb at a
15 ratio of NO:Hb in the range of 1:100 to 1:2, with a ratio of NO/heme of approximately 1:10 being preferred. If the ratio of NO:heme is increased, to a NO:Hb of about 2 (at which Hb is still in the T (deoxy) state), in the absence of an electron acceptor/free radical scavenger, NO is
20 released from the β heme as NO^- , with oxidation of the heme iron to form metHb. The product solution can be used as a blood substitute or a therapeutic NO donor. NO^- can protect from N-methyl-D-glutamate-mediated brain injury in stroke; this effect has not been found for NO.

25 Nitrosylhemoglobin belongs to a broader class of nitrosyl-heme-containing donors of NO which can be administered to an animal or human for the delivery of NO to tissues. Nitrosyl-heme-containing donors of NO include, for example, the nitrosated ("nitrosated" as defined
30 herein) hemoglobins nitrosylhemoglobin and SNO-nitrosylhemoglobin, nitrosyl-heme, and substituted forms of hemoglobin in which a different metal, (e.g., Co^{++} , Mg^{++} , Cu^{++}) is substituted for the heme iron, or nitrosyl-porphyrins are substituted for the heme.

Applicants teach physiologically significant results that provide a rationale for NO donors to be attached to Hb. Such derivatized Hbs can themselves serve as NO-donating therapeutics and can ameliorate the side effects of underivatized Hb administered as a blood substitute, for example. At one time, it had been thought that there would be no use for these compounds, because it was thought that NO released by the Hb would immediately be scavenged by the heme. It had been thought also that the released NO would oxidize Hb and limit oxygen delivery. The same rationale has previously limited the administration of NO donors, such as nitroglycerin and nitroprusside, because they had been thought to cause the formation of methHb.

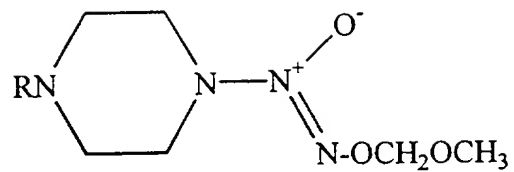
Preferably, NO-donors to be covalently attached to hemoglobin are relatively long-lived and have at least one functional group that can be used for the chemical attachment to hemoglobin. Examples of NO-donors include nitroprusside, nitroglycerin, nitrosothiols, and the diazeniumdiolate class of compounds (also called "NONOates") having structure 1.



A variety of these compounds have been synthesized that, in their anionic form, release NO without activation at physiological pH (Keefer, L.K. et al., *Am. Chem. Soc. Symposium Ser.* 553:136-146 (1994); Hanson, S.R. et al., *Adv. Pharmacol.* 34:383-398 (1995)). Systemic administration can result in system-wide effects, according to equation 1. However, attachment to hemoglobin can be used to produce tissue-selective delivery of NO and oxygen. For instance, covalently esterified NO-donors can be activated predominantly in the liver. Different NO donors

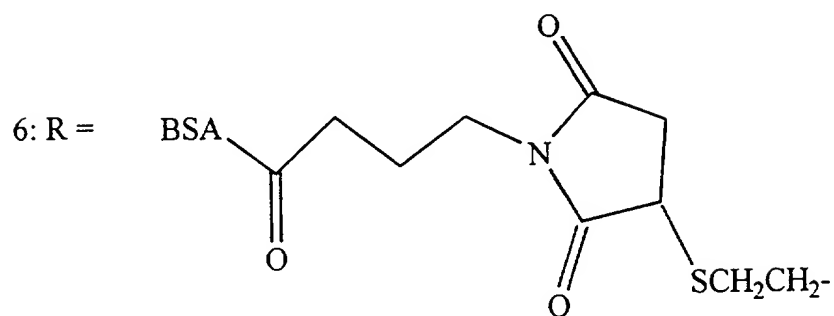
can be chosen to be linked to hemoglobin for different controlled release rates of NO from Hb.

Compound 4, for example, is a diazeniumdiolate with a half-life for NO release, at 37°C and pH 7.4, of approximately two weeks. It can be converted to its nucleophilic N-4 mercaptoethyl derivative, compound 5. Hemoglobin can be activated toward coupling reactions by reacting it with γ -maleimidobutyric acid N-hydroxysuccinimide ester. Compound 5 can then be covalently attached to the activated hemoglobin through its maleimide functionality. The adduct, 6, can generate NO steadily over several days in pH 7.4 phosphate buffer at 37°C. This would alleviate side effects of underivatized blood substitutes, for example.



4: R = H-

5: R = HSCH₂CH₂-



Nitric oxide synthase (NOS) conjugated to Hb can reload NO onto the hemes, facilitating delivery of NO to the tissues. NOS of neurons is preferable for this conjugated Hb because the neuronal NOS responds to oxygen
5 tension. At low oxygen tension, the neuronal NOS produces more NO; at high oxygen tension, NOS produces less NO. This form of NOS will efficiently reload NO onto the heme when Hb is deoxygenated. NOS-Hb conjugates can be used when a blood substitute is indicated, and especially when
10 an ischemic injury or condition is present.

Biologically compatible electron acceptors, are well known in the art and include, but are not limited to, superoxide dismutase and the oxidized forms of nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine
15 dinucleotide phosphate (NADP^+), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), ascorbate, dehydroascorbate and nitroxide spin traps. One or more electron acceptors can be conjugated to Hb molecules, and can facilitate the conversion of the nitrosyl-Hb-electron
20 acceptor form to the SNO-Hb-electron acceptor form by accepting the electron lost by NO in its transfer, in the form of NO^+ or as RSNO^\bullet , to a $\beta 93\text{Cys}$ thiol group.

Nitroxides are one such class of electron acceptors which also act as free radical scavengers. Nitroxides are
25 stable free radicals that have been shown to have antioxidant catalytic activities which mimic those of superoxide dismutase (SOD), and which when existing *in vivo*, can interact with other substances to perform catalase-mimic activity. Nitroxides have been covalently
30 attached to hemoglobin. See Hsia, J-C., U.S. Serial No. 5,591,710, the contents of which are incorporated by reference in their entirety. See also Liebmann, J. *et al.*, *Life Sci.* 54:503-509 (1994), describing nitroxide-conjugated bovine serum albumin and differential nitroxide

concentrations among the different organs of mice tested with the conjugate.

Methods for chemically attaching superoxide dismutase (SOD) to Hb are known in the art. For example, see Quebec, 5 E.A. and T.M. Chang, *Artif. Cells Blood Substit. Immobil. Biotechnol.* 23:693-705 (1995) and D'Agnillo, F. and Chang, T.M., *Biomater. Artif. Cells Immobilization Biotechnol.* 21:609-621 (1993). SOD attached to nitrosylhemoglobin can drive the reaction in which NO is transferred from the heme 10 to thiol, by serving as an electron acceptor.

Like NO, CO is known to have vasodilator effects. (See Zakhary, R. et al., *Proc. Natl. Acad. Sci USA* 93:795-798 (1996).) A solution of deoxyhemoglobin can be derivatized with CO by exposing it to purified CO gas in 15 solution, until the desired extent of CO-bound Hb is reached. CO-derivatized Hb can be administered as a blood substitute or co-administered with other heme-based blood substitutes to alleviate the effects (e.g., hypertension, intestinal pain and immobility) of underivatized 20 hemoglobin. Hemoglobins can be derivatized to the extent necessary to overcome constrictor effects, for example to a ratio of CO/Hb in the range of approximately 0.1% to 10%.

Because the α subunits lack thiol groups to serve as NO⁺ acceptors from the heme, a blood substitute comprising 25 α chains, for example in the form of dimers or tetramers, can be made which has different properties from a blood substitute comprising β chains alone, or comprising a combination of α and β chains. A blood substitute comprising α chains of hemoglobin can be administered to an 30 animal or to a human patient to alleviate a condition characterized by the effects caused by NO, for example, in hypotensive shock.

β chains, unlike α chains, serve as active donors of NO to the tissues, rather than traps for NO. A blood 35 substitute comprising β chains, for example in the form of

β dimers or tetramers, can be made. Such a blood substitute can be used in conditions where it is desired to deliver oxygen as well as NO to tissues, for example, in angina and other ischemic conditions.

- 5 Methods are known by which hemoglobin can be separated into its α and β subunits and reconstituted. Separated, heme-free, alpha- and beta-globins have been prepared from the heme-containing alpha and beta subunits of hemoglobin. (Yip, Y.K. et al., *J. Biol. Chem.* 247:7237-7244 (1972)).
- 10 Native human hemoglobin has been fully reconstituted from separated heme-free alpha and beta globin and from hemin. Preferably, heme is first added to the alpha-globin subunit. The heme-bound alpha globin is then complexed to the heme-free beta subunit. Finally, heme is added to the
- 15 half-filled globin dimer, and tetrameric hemoglobin is obtained (Yip, Y.K. et al., *Proc. Natl. Acad. Sci. USA* 74:64-68 (1997)).

- The human alpha and beta globin genes reside on chromosomes 16 and 11, respectively. Both genes have been
- 20 cloned and sequenced, (Liebhaber, et al., *Proc. Natl. Acad. Sci. USA* 77:7054-7058 (1980) (alpha-globin genomic DNA); Marotta, et al., *J. Biol. Chem.* 252:5040-5053 (1977) (beta globin cDNA); Lawn, et al., *Cell* 21:647 (1980) (beta globin genomic DNA)).

- 25 Recombinant methods are available for the production of separate α and β subunits of hemoglobin. Nagai and Thorgerson, (*Nature* 309:810-812 (1984)) expressed in *E. coli* a hybrid protein consisting of the 31 amino terminal residues of the lambda cII protein, an Ile-Glu-Gly-Arg
- 30 linker, and the complete human beta globin chain. They cleaved the hybrid immediately after the linker with blood coagulation factor Xa, thus liberating the beta-globin chain. Later, (Nagai, K. et al., *Proc. Natl. Acad. Sci. USA* 82:7252-7255 (1985)) took the recombinant DNA-derived
- 35 human beta globin, naturally derived human alpha globin,

and a source of heme and succeeded in producing active human hemoglobin.

An efficient bacterial expression system for human alpha globin was reported. (GB 8711614, filed May 16, 5 1987; see also WO 88/09179). This led to the production of wholly synthetic human hemoglobin by separate expression of the insoluble globin subunits in separate bacterial cell lines, and *in situ* refolding of the chains in the presence of oxidized heme cofactor to obtain tetameric hemoglobin. 10 A synthetic human hemoglobin has been produced in yeast cells (EP 700997A1, filing date 10.05.1990).

The properties of hemoglobin have been altered by specifically chemically crosslinking the alpha chains between the Lys99 of alpha 1 and the Lys99 of alpha 2. 15 (Walder, U.S. 4,600,531 and 4,598,064; Snyder, et al., *Proc. Natl. Acad. Sci USA* 84:84 7280-7284 (1987); Chatterjee, et al., *J. Biol. Chem.* 261:9927-9937 (1986)). This chemical crosslinking was accomplished by reacting bis (3,5-dibromosalicyl) fumarate with deoxyhemoglobin A in the 20 presence of inositol hexaphosphate. The beta chains have also been chemically crosslinked. (Kavanaugh, M.P. et al., *Biochemistry* 27:1804-1808 (1988)). These linking methods can be adapted to methods of producing α or β dimers or other multimers, or for the crosslinking of other 25 polypeptides to the α and β chains. (For further methods to derivatize proteins and to conjugate proteins, see Hermansoh, G.T., *Bioconjugate Techniques*, Academic Press, 1996.)

The term hemoglobin or Hb as used herein includes 30 variant forms such as mutant forms, chemically modified forms, genetically altered forms, such as fusion proteins, and truncated forms. It also includes Hbs of all animal species and variant forms thereof. The biological and/or chemical properties of these variant Hbs may be different

from those of hemoglobins which are found naturally occurring in animals.

It will be appreciated that NO exists in biological systems not only as nitric oxide gas, but also in various
5 redox forms and as biologically active adducts of nitric oxide such as S-nitrosothiols, which can include S-nitrosoproteins, S-nitroso-amino acids and other S-nitrosothiols (Stamler, J.S. *Cell* 78:931-936 (1994)).

A blood substitute can be a biologically compatible
10 liquid which performs one or more functions of naturally occurring blood found in a mammal, such as oxygen carrying and/or delivery, NO carrying and/or delivery, and the scavenging of free radicals. A blood substitute can also comprise one or more components of such a liquid which,
15 when infused into a mammal, perform one or more functions of naturally occurring blood. Examples of blood substitutes include preparations of various forms of hemoglobin. Such preparations may also include other biologically active components, such as a low molecular
20 weight thiol, nitrosothiol or NO donating agents, to allow transnitrosation.

The compounds and therapeutic preparations of this invention to be used in medical treatment are intended to be used in therapeutically effective amounts, in suitable
25 compositions, which can be determined by one of skill in the art. Modes of administration are those known in the art which are most suitable to the affected site or system of the medical disorder. Intravenous infusion is a preferred mode of administration of various forms of
30 hemoglobin to be used as a blood substitute. Suitable compositions may include carriers, stabilizers or inert ingredients known to those of skill in the art, along with biologically active component(s).

The term "therapeutically effective amount," for the
35 purposes of the invention, refers to the amount of

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nitrosated Hb and/or nitrosating agent which is effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges for effective amounts of each compound to be administered is within the skill of one in the art. Research animals such as dogs, baboons or rats can be used to determine dosages. Generally, dosages required to provide effective amounts of the composition or preparation, and which can be adjusted by one of ordinary skill in the art, will vary, depending on the age, health, physical condition, sex, weight, extent of disease of the recipient, frequency of treatment and the nature and scope of the desired effect. Dosages for a particular patient can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol). For example, dose response experiments for determining an appropriate dose of a heme-based blood substitute can be performed to determine dosages necessary to produce a physiological concentration of approximately 1 nM to 100 μ M heme. Suitable pharmaceutical carriers can be combined with active ingredients employed in a therapeutic composition, if necessary.

The present invention is further and more specifically illustrated in the following examples, which are not intended to be limiting in any way.

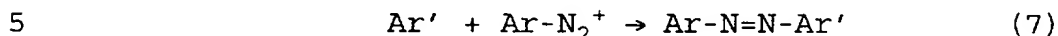
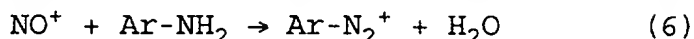
EXEMPLIFICATION

Materials and Methods for Assays

Determination of R-S-NO Concentration

The concentration of R-S-NO groups in a sample is based on the method reported in Saville, *Analyst* 83:670-672 (1958). The quantification of the NO group, displaced from the thiol by mercuric ion, forms the basis of this highly

sensitive method. The detection limit is in the range of 0.1-0.5 μM .



As shown (equations 5-7), the reaction proceeds in two steps. First, NO^+ is displaced from the RSNO by mercuric ion and reacts, under acidic conditions, with sulfanilamide (Ar-NH_2). In a second step, the diazonium salt (which is
10 formed in amounts equivalent to the thionitrite) is then coupled with the aromatic amine, N-(1-naphthyl)-ethylenediamine (Ar'), to form an intensely colored azo dye which can be measured at 540 nm ($\epsilon \sim 50,000 \text{ M}^{-1} \text{ cm}^{-1}$). The same assay performed with the mercuric salt omitted allows
15 for the simultaneous detection of nitrite. In principle, the second part of the Saville procedure is analogous to the classical Griess reaction for the detection of nitrite.

The procedure is as follows:

Solution A: sulfanilamide 1% dissolved in 0.5 M HCl.

20 Solution B: same solution as used in A to which 0.2% HgCl_2

Solution C: 0.02% solution of N-(1-naphthyl)-ethylenediamine dihydrochloride dissolved in 0.5 M HCl.

A given volume (50 μl -1ml) of the sample to be assayed is added to an equivalent volume of solution A and solution
25 B. The two samples are set aside for 5 minutes to allow formation of the diazonium salt, after which an equivalent volume of solution C is added to each mixture. Color formation, indicative of the azo dye product, is usually complete by 5 minutes. The sample absorbance is then read
30 spectrophotometrically at 540 nm. The RSNO is quantified as the difference in absorbance between solution B and A. (i.e. B - A). In the event that the background nitrite

concentration is high (i.e. increased background in A), the accuracy of the measurement can be increased by the addition of an equivalent volume of 0.5% ammonium sulfamate in acid (45 mM) 5 minutes prior to the addition of
5 sulfanilamide. The nitrous acid in solution reacts immediately with excess ammonium sulfamate to form nitrogen gas and sulfate.

Concentrations of thiol greater than 500 μ M in samples may interfere with the assay if nitrite is also present at
10 micromolar concentration. Because nitrite will nitrosate indiscriminantly under the acidic conditions employed, thiols will effectively compete for reaction with sulfanilamide (present at 50 mM in this assay) as their concentration approaches the millimolar range. This will
15 lead to artifactual detection of RSNO. The problem can be avoided by (1) keeping the ratio of thiol to sulfanilamide < 0.01, (2) first alkylating thiols in the solution, or (3) adding free thiols to standards to correct for the potential artifact.

20 *Assay for S-nitrosohemoglobin and Nitrosyl(FeII)-Hemoglobin*

A highly sensitive photolysis-chemiluminescence methodology was employed, which had been used for measuring RSNOs (S-nitrosothiols) in biological systems (Gaston, B., et al., *Proc. Natl. Acad. Sci. USA* 90:10957-10961 (1993);
25 Stamler, J.S., et al., *Proc. Natl. Acad. Sci USA* 89:7674-7677 (1992)). The method involves photolytic liberation of NO from the thiol, which is then detected in a chemiluminescence spectrometer by reaction with ozone. The same principle of operation can be used to cleave (and
30 measure) NO from nitrosyl-metal compounds (Antonini, E. and Brunori, M. *In Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York, pp. 29-31 (1971)). With adjustment of flow rates in the photolysis cell, complete photolysis of the NO ligand

of Hb(FeII)NO is achieved. Standard curves derived from synthetic preparations of SNO-Hb, Hb(FeII)NO, and S-nitrosoglutathione were linear ($R > 0.99$), virtually superimposable, and revealing of sensitivity limits of approximately 1 nM. Two analytical criteria were then found to reliably distinguish SNO-Hb from Hb(FeII)NO: 1) signals from SNO-Hb were eliminated by pretreatment of samples with 10-fold excess HgCl_2 , while Hb(FeII)NO was resistant to mercury challenge; and 2) treatment of SNO-Hb with HgCl_2 produced nitrite (by standard Griess reactions) in quantitative yields, whereas similar treatment of Hb(FeII)NO did not. UV/VIS spectroscopy confirmed that NO remained attached to heme in the presence of excess HgCl_2 .

We linked a photolysis cell directly to the reaction chamber and detector portion (bypassing the pyrolyzer) of a chemiluminescence apparatus (model 543 thermal energy analyzer, Thermedix, Woburn MA). A sample (5 to 100 μl) is either introduced directly or introduced as a chromatographic effluent from an attached high-performance liquid or gas chromatography system into the photolysis cell (Nitrolite, Thermedix, Woburn MA). This cell consists of a borosilicate glass coil (3 m x 0.64 cm o.d. x 1 mm i.d., turned to a diameter of 6 cm and a width of 12 cm). The sample is introduced with a purge stream of helium (5 liters/min) and then irradiated with a 200-W mercury-vapor lamp (vertically mounted in the center of the photolysis coil on Telfon towers). The effluent from the photolysis coil is directed to a series of cold traps, where liquid and gaseous fractions less volatile than nitric oxide (such as nitrite and nitrate) are removed. Nitric oxide is then carried by the helium stream into the chemiluminescence spectrometer, in which free nitric oxide is detected by reaction with ozone. Signals are recorded on a digital integrator (model 3393A, Hewlett-Packard). Flow rates and illumination levels in the photolysis cell were designed to

result in complete photolysis of the S-N bond of S-nitrosothiols, as confirmed by analysis of effluent from the cell according to the method of Saville (Saville, B., *Analyst* 83:670-672 (1958)).

- 5 To determine what fraction of the total nitric oxide detected in samples was derived from S-nitrosothiols, several control measurements were performed. Mercuric ion was used to displace nitric oxide selectively from the S-nitrosothiols (Saville, B., *Analyst* 83:670-672 (1958)).
- 10 Comparison of measured nitric oxide concentrations from samples alternatively pretreated or not pretreated with HgCl_2 ensured that nitric oxide obtained by photolysis was derived specifically from S-nitrosothiols. Similarly, as an added measure of confirmation, we distinguished between
- 15 S-nitrosothiols and free nitric oxide by comparing nitric oxide concentrations in samples alternatively exposed or not exposed to photolyzing illumination.

Methods for Spectrophotometric Experiments and Nitrosylhemoglobin Formation, Examples 14-20

- 20 Purified human HbA_0 was obtained from Apex Biosciences (Antonini, E. and Brunori, M. *In Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York (1971)). The spectrophotometer used was a Perkin Elmer UV/vis
- 25 Spectrometer Lambda 2S. All measurements were made at 23°C in a sealed quartz cuvette to which all additions were made. Deoxygenation was achieved by argon passage through a Hb solution within a sealed quartz cuvette. The degree of deoxygenation can be measured by UV/vis spectrum.
- 30 Nitrosylation of hemes is achieved by addition of purified NO gas to deoxyHb and the products quantitated by the extinction coefficient per Antonini and Brunori, *supra*.

Example 1: Interactions of NO and RSNO with Hb

It was observed that naturally occurring N-oxides, such as NO and RSNOs (Gaston, B., et al., *Proc. Natl. Acad. Sci. USA* 90:10957-10961 (1993); Scharfstein, J.S., et al., *J. Clin. Invest.*, 94:1432-1439 (1994); Clancy, R.M., et al., *Proc. Natl. Acad. Sci USA*, 91:3680-3684 (1994)), differed markedly in their reactions with Hb. NO bound very rapidly to deoxyHb (Hb[FeII]), forming relatively stable Hb[FeII]NO complexes (Figure 1A), and converted oxyHb (Hb[FeII]O₂) to methemoglobin (Hb[FeIII]) and nitrate (Figure 1B), confirming previous reports (Olson, J.S., *Meth. Enzymol.* 76:631-651 (1981); Toothill, C., *Brit. J. Anaesthy.* 39:405-412 (1967)). In contrast, RSNOs were found to participate in transnitrosation reactions with sulfhydryl groups of Hb, forming S-nitrosohemoglobin (SNO-Hb), and did not react with the heme centers of either deoxyHb or Hb(FeII)O₂ (Figures 1C and 1D).

A. Interaction of NO with deoxyHb

Conversion of deoxyHb (Hb[FeII]) to Hb(FeII)NO is observed upon incubation of Hb(FeII) with increasing concentrations of nitric oxide. See Figure 1A. a. Deoxy Hb. b, c, d. Reaction mixtures of NO and Hb(FeII) in ratios of 1:1, 2:1 and 10:1, respectively. The reaction product Hb(FeII)NO formed essentially instantaneously on addition of NO (i.e. within instrument dead time).

B. Interaction of NO with oxyHb

Conversion of oxyHb (Hb[Fe[II]O₂) to methHb (HbFe[III]) is observed upon incubation of oxyHb with increasing concentrations of NO. See Figure 1B. a. oxy Hb. b, c, d. Reaction mixtures containing NO and oxyHb in ratios of 1:1, 2:1 and 10:1, respectively. Methemoglobin formation occurred instantaneously on addition of NO (i.e. within instrument dead time).

C. Interaction of S-nitrosothiols with deoxyHb

Conversion of Hb(FeII) to SNO-Hb(FeII) is observed upon incubation of either GSNO (shown) or S-nitrosocysteine (CYSNO) with deoxy Hb. There is little (if any)

- 5 interaction of RSNO with the heme functionalities of Hb. See Figure 1C. a. deoxyHb. b, c, d. Reaction mixtures of GSNO and Hb(FeII) in ratios of 1:1, 2:1 and 10:1, respectively. Spectra were taken after 60 min of incubation in b, c, and 15 min in d. Further analysis of
10 reaction products revealed the formation of moderate amounts of SNO-Hb in all cases. Yields of SNO-Hb (S-NO/Hb) in b, c, and d at 60 min were 2.5%, 5% and 18.5%, respectively. (See Figure 1D and Figure 2A.)

D. Interaction of S-nitrosothiols with oxyHb

- 15 Conversion of Hb(FeII)O₂ to SNO-Hb(FeII)O₂ is observed upon incubation of either GSNO (shown) or CYSNO with oxyHb. There is little (if any) reaction of GSNO (or CYSNO) at the heme centers of Hb(FeII)O₂. Specifically, the capacity for O₂ binding to heme is unaffected by RSNOS. See Figure 1D.
20 a. oxyHb. b, c, d. Reaction mixtures of GSNO and oxyHb in ratios of 1:1, 2:1, and 10:1, respectively. Spectra were taken after 60 min of incubation in the spectrophotometer. Further analysis of reaction products revealed the formation of SNO-Hb in all cases. Yields of SNO-Hb in
25 spectra b, c and d were 5%, 10% and 50% (S-NO/Hb), respectively. In 5 other determinations, the yield of S-NO/Hb was 0.37 ± 0.06 using GSNO (pH 7.4, 10-fold excess over Hb) and ~ 2 SNO/tetramer (1.97 ± 0.06) using CYSNO (*vida infra*). These last data are in agreement with
30 reports that human HbA contains 2 titratable SH groups.

Methods

Human HbA₀ was purified from red cells as previously described (Kilbourn, R.G., et al., Biochem. Biophys. Res.

Comm., 199:155-162 (1994)). Nitric oxide solutions were rigorously degassed and purified according to standard procedure (Beckman, J. S., et al., *Methods in Nitric Oxide Research*, Feelisch and Stamler, eds., Wiley Chichester, U.K. (1996)) and saturated solutions were transferred in air tight syringes. Deoxygenation of Hb was achieved by addition of excess dithionite (NO studies) or by reduction of Hb(FeII)O₂ through evacuation in Thunberg tubes (RSNO studies; as RSNOs react with dithionite). RSNOs were synthesized as previously described (Gaston, B., et al., (1993); Arnette, D.R. and Stamler, J.S., *Arch. Biochem. Biophys.* 318:270-285 (1995)) Incubations with HbA₀ were made in phosphate buffer, pH 7.4, 0.5 mM EDTA. Quantifications of SNO-Hb were made according to the method of Saville (Gaston, B., et al., (1993); Stamler, J.S., et al., *Proc. Natl Acad. Sci. USA*, 90:444-448 (1992)) after purification of protein with Sephadex G-25 columns. The Saville method, which assays free NO_x in solution, involves a diazotization reaction with sulfanilamide and subsequent coupling with the chromophore N-(naphthyl)ethylenediamine. No low molecular weight S-NO complexes survived this purification and all activity was protein precipitable. The reactions and spectra were carried out using a Perkin Elmer UV/Vis Spectrometer, Lambda 2S.

25 Example 2: Allosteric Function of O₂ in Regulation of Hb S-nitrosylation

Oxygenation of Hb is associated with conformational changes that increase the reactivity of cysβ93 to alkylating reagents (Garel, C., et al., *J. Biochem.*, 123:513-519 (1982); Jocelyn, P.C., *Biochemistry of the SH Group*, Academic Press, London, p.243 (1972); Craescu, C.T., et al., *J. Biol. Chem.*, 261:14710-14716 (1986)). The physiological importance of this effect was not established. It was observed here that rates of S-

nitrosation of Hb were markedly dependent on conformational state. In the oxy conformation (R state), S-nitrosation was more rapid than in the deoxy conformation (T state) (Figure 2A). The rate of S-nitrosation was accelerated in both conformations by alkaline conditions (i.e., rate at pH 9.2 > pH 7.4), which would tend to expose the cys β 93 that is otherwise screened from reaction by the C-terminal histidine 146 β . The salt bridge (asp β 94 --- his β 146) tying down the histidine residue is loosened at high pH. These data suggest that the increase in thiol reactivity associated with the R state derives, at least in part, from improved NO access rather than a conformation-induced change in pK.

A. Oxygenation accelerates S-nitrosylation of Hb.

Rates of Hb S-nitrosation by S-nitrosocysteine (CYSNO) are faster in the oxy conformation (Hb[FeII]O₂) than in the deoxy state (Hb[FeII]).

Methods

Incubations were performed using 10-fold excess CYSNO over protein (50 μ M) in aerated 2% borate, 0.5 mM EDTA (oxyHb), or in a tonometer after rapid O₂ evacuation (deoxyHb). At times shown in Figure 2A, samples were rapidly desalted across G-25 columns (preequilibrated with phosphate buffered saline, 0.5 mM EDTA, pH 7.4) to remove CYSNO, and analyzed for SNO-Hb by the method of Saville (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA*, 89:444-448 (1992)).

B. Deoxygenation accelerates denitrosylation of Hb

Rates of RSNO decomposition (and transfer) are much faster in the deoxy conformation [SNO-Hb(FeII)] than in the oxy state [SNO-Hb(FeII)O₂]. The decomposition of SNO-Hb(FeII) is further accelerated by the presence of excess

glutathione. Within the dead time of measurements according to this method (~15 seconds), a major fraction of SNO-Hb(FeII) was converted to GSNO.

Methods

5 Hbs in PBS (0.5 mM EDTA, pH 7.4) were incubated in air (oxy) or in a tonometer previously evacuated of O₂ (deoxy). SNO-Hb(FeII)O₂ decomposition was determined by the method of Saville (Saville, B., *Analyst* 83:670-672 (1958)). Spontaneous decomposition of SNO-Hb(FeII) was followed
10 spectrophotometrically by formation of Hb(FeII)NO. Transnitrosation reactions with glutathione were performed by addition of 100-fold excess glutathione over protein (50 μM), immediate processing of the reaction mixture under anaerobic conditions followed by rapid TCA precipitation,
15 and analysis of RSNO in the supernatant. Rates of NO group transfer were too rapid to measure accurately by the standard methods used in this study.

Example 3: NO-related Interactions with Cysteine Residues of Hb in Physiological Systems

20 Given that Hb is largely contained within red blood cells, potential mechanisms by which S-nitrosation of intracellular protein might occur were explored. Incubation of oxygenated rat red blood cells with S-nitrosocysteine resulted in very rapid formation of
25 intracellular SNO-Hb(FeII)O₂ (Figure 3A). Rapid oxidation of Hb was not observed under these conditions. Intraerythrocytic SNO-Hb also formed when red blood cells were treated with S-nitrosohomocysteine or S-nitrosocysteinyglycine, but not with S-nitrosoglutathione
30 (GSNO). Thus, erythrocyte access of RSNOs is thiol group specific. Exposure of oxygenated red blood cells to NO resulted primarily in methHb formation.

Endothelium-derived relaxing factor (EDRF) and hemoglobin

Hb-mediated inhibition of endothelium-dependent relaxations is commonly used as a marker of NO responses. Inasmuch as reactions with either metal or thiol centers of Hb should lead to attenuated NO/EDRF (endothelium-derived relaxing factor) responses, experiments were performed to elucidate the molecular basis of inhibition. Hb preparations in which $\beta 93$ thiol groups had been blocked with N-ethylmaleimide (NEM) or the hemes blocked by cyanmet (FeIIICN)-derivitization were studied in an aortic ring bioassay, and their activities were compared with that of native Hb. Both cyanmet-Hb and NEM-Hb caused increases in vessel tone and attenuated acetylcholine (EDRF)-mediated relaxations (Figure 3B). However, native Hb was significantly more effective than either of the modified Hb preparations (Figure 3B). Taken in aggregate, these studies suggest that both the thiol and metal groups of Hb contribute to its NO-related activity. To verify formation of an S-nitrosothiol in Hb, a bioassay was used in which 2 cm segments of thoracic aorta were interposed in Tygon tubing, through which 3 cc of Krebs solution containing Hb ($4 \mu\text{M}$) and ACh ($2 \mu\text{M}$) were circulated by roller pump ($1.5 \text{ cc/min} \times 5 \text{ min}$). Analysis of the effluent (Gaston, B., et al., (1993)) revealed the formation of SNO-Hb ($20 \pm 4 \text{ nM}$) in 5 of 5 experiments.

A. S-nitrosation of intraerythrocytic Hb

Incubation of rat erythrocytes with S-nitrosocysteine (equimolar to heme (5 mM); phosphate buffer pH 7.4, 25°C) leads to rapid formation of intracellular SNO-Hb(FeII)O₂. MetHb does not form rapidly. Separation of intracellular RSNOs across G-25 columns reveals that only a small percentage exists as low molecular weight S-nitrosothiol (e.g. GSNO) at most time points. By 60 min, 3 of the 4 available SH groups of Hb were S-nitrosated (note that rat

Hb contains 4 reactive SH groups). See Figure 3A. Inset shows spectra of SNO-Hb isolated from rat erythrocytes and related analyses. Spectrum A is that of SNO-Hb isolated from erythrocytes following G-25 chromatography. Treatment
5 of A with dithionite results in reduction of the S-NO moiety, liberating free NO which is autocaptured by deoxy Hb, forming Hb(FeII)NO (note that dithionite simultaneously deoxygenates Hb) (spectrum C). This spectrum (C) reveals a stoichiometry of ~3 S-NOs per tetramer. The spectrum of
10 Hb(FeII)NO containing 4 NO's per tetramer is shown for comparison (inset, spectrum B).

Methods

At shown intervals, red blood cells were pelleted rapidly by centrifugation, washed three times, lysed in
15 deionized water at 4°C, and the cytosolic fraction subjected to rapid desalting across G-25 columns. Intracellular SNO-Hb was measured by the method of Saville (Gaston, B., et al., (1992); Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA*, 89:444-448 (1992)), and confirmed
20 spectroscopically (inset of Figure 3A) as described above.

B. Molecular Basis of EDRF/Hb Interaction.

The effects of native Hb on EDRF responses were compared with Hb preparations in which the thiol or heme centers had been blocked by alkylation or cyanmet
25 derivitization, respectively. All preparations of Hb elicited contractions; however, those of native Hb (in which both SH and metal centers are free for interaction) were most pronounced. See Figure 3B. Likewise, acetylcholine (ACh) mediated relaxations were most
30 effectively inhibited by native Hb. Relaxations were inhibited to lesser degrees by cyanmet Hb (CN-Hb) (in which hemes were blocked from reaction) and NEM-Hb (in which thiol groups were alkylated by N-ethylmaleimide). See

Table 1. These data illustrate that both heme and $\beta 93\text{SH}$ groups of Hb contribute to reversal of EDRF responses. Direct measurement of SNO-Hb, formed from EDRF under similar conditions, is described in Example 8.

5 Methods

Descending rabbit thoracic aorta were cut into 3 mm rings and mounted on stirrups attached to force transducers (model FT03, Grass Instruments, Quincy, MA) for measurement of isometric tone. The details of this bioassay system have been previously described (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA*, 89:444-448 (1992)). Cyanmet Hb was prepared from human HbA according to published protocols (Kilbourn, R.G. et al. *Biochem. Biophys. Res. Comm.*, 199:155-162, (1994)). Alkylation of HbA with N-ethylmaleimide was followed by desalting across G-25 Sephadex to remove excess NEM. Removal of unmodified Hbcys $\beta 93$ was achieved by passage through Hg-containing affinity columns. The alkylation of free SH groups was verified using 5,5'-dithio-bis[2-nitrobenzoic acid].

Table 1

ADDITIONS	% INCREASE IN TENSION (†)	% ACh RELAXATION (†)
Hb (1 μ M)	40.8 ± 2.3 (n=7)	31.9 ± 6.9 (n=7)
NEM-Hb (1 μ M)	29.4 ± 1.3 ** (n=7)	60.5 ± 3.9 * (n=7)
CN-Hb (1 μ M)	12.9 ± 3.0 ** (n=6)	80.7 ± 1.0 ** † (n=4)
ACh (1 μ M)		98.3 ± 0.6 (n=10)
*, P<0.01; **, P<0.001, Compared to Hb; †, P<0.001, Compared to ACh		

Example 4: Transduction of SNO-Hb Vasoactivity

Arterial red blood cells contain two physiologically important forms of hemoglobin: Hb(FeII)O₂ and Hb(FeIII) (Antonini, E. and Brunori, M. *In Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York, pp. 29-31 (1971)).

Arterial-venous differences in the S-nitrosothiol content of intraerythrocytic Hb suggest that the NO group is released during red cell transit. Such findings raise the possibility of functional consequences, perhaps influenced by the redox state of heme and its occupation by ligand. SNO-Hb(FeII)O₂ was found to possess modest NO-like activity when tested in a vascular ring bioassay. Specifically, the contraction elicited by SNO-Hb(FeII)O₂ was less than that of native Hb(FeII)O₂, indicating that S-nitrosation partially reverses the contractile effects of Hb (Figure 4A). By comparison, SNO-Hb(FeIII) was found to be a vasodilator (Figure 4A). Notably, free NO was devoid of relaxant activity in the presence of Hb(FeII)O₂ or Hb(FeIII) (not shown).

Red blood cells contain millimolar concentrations of glutathione. As equilibria among RSNOs are rapidly established through RSNO/thiol exchange (Arnette, D.R. and Stamler, J.S., *Arch. Biochem. Biophys.*, 318:279-285 (1995)), the vasoactivity of SNO-Hb was reassessed in the presence of glutathione. Figure 4B illustrates that glutathione potentiated the vasodilator activity of both SNO-Hb(FeII)O₂ and SNO-Hb(FeIII). GSNO formation under these conditions (confirmed chemically and in bioassay experiments) appeared to fully account for this effect. Further kinetic analyses revealed that transnitrosation involving glutathione was more strongly favored in the equilibrium with SNO-Hb(FeIII) than SNO-Hb(FeII)O₂ (Figure 4C). Given the findings of steady-state levels of SNO-Hb in red blood cells (Table 2 and Figure 3A), these results suggest that 1) the

equilibrium between naturally occurring RSNOs and Hb(cys β 93) lies toward SNO-Hb under physiological conditions; 2) that transnitrosation reactions involving SNO-Hb and GSH are likely to occur within red blood cells
5 (in these studies, low molecular weight RSNOs have been found in erythrocytes loaded with SNO-Hb); and 3) that oxidation of the metal center of Hb shift the equilibrium toward GSNO, thereby potentially influencing bioactivity.

Additional mechanisms of NO group release from SNO-Hb
10 were sought. Arterial-venous differences in levels of SNO-Hb raised the possibility that S-NO bond stability may be regulated by the changes in Hb conformation accompanying deoxygenation. To test this possibility, the rates of NO group release from SNO-Hb(FeII)O₂ and SNO-Hb(FeIII) were
15 compared. Deoxygenation was found to enhance the rate of SNO-Hb decomposition (Figure 2B). These rates were accelerated greatly by glutathione in a reaction yielding GSNO (Figure 2B). The results illustrate that O₂-metal interactions influence S-NO affinity, and suggest a new
20 allosteric function for Hb.

For SNO-Hb to be of physiological importance it must transduce its NO-related activity across the erythrocyte membrane. This possibility was explored by incubating erythrocytes containing SNO-Hb in physiologic buffer, and
25 measuring the accumulation of extracellular RSNOs over time. Figure 4D illustrates that red blood cells export low molecular weight (trichloroacetic acid precipitable) S-nitrosothiols under these conditions. Importantly, the degree of hemolysis in these experiments was trivial
30 (<0.5%), and correction for lysis did not significantly impact on rates of RSN0 release. These results establish that an equilibrium exists between low molecular weight and protein RSNOs within the red cell, and that intracellular location is unlikely to be a limiting factor in the

transduction of such NO-related activity to the vessel wall.

A. Concentration-effect responses of different SNO-Hb preparations.

5 Contractile effects of Hb(FeII)O_2 (\blacktriangle) are shown to be partially reversed by S-nitrosation (SNO-Hb[FeII]O_2 (\blacksquare); $P = 0.02$ by ANOVA vs Hb(FeII)O_2) (See Figure 4A.). Oxidation of the metal center of SNO-Hb (SNO-Hb[FeIII] (\bullet)) converts the protein into a vasodilator ($P < 0.0001$ by ANOVA vs.

10 SNO-Hb[FeII]O_2), with potency comparable to that of other S-nitrosoproteins (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA*, 89:444-448 (1992)). The contractile properties of Hb(FeIII) are shown for comparison (\square); $n = 6-17$ for each data point.

15 Methods

Details of the vessel ring bioassay have been published (Stamler, J.S., et al., *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992)). SNO-Hb(FeII)O_2 preparations were synthesized with 10-fold excess S-nitrosocysteine (CYSNO)

20 over Hb(FeII)O_2 protein (2% borate, 0.5 mM EDTA, ~15 min incubation), after which desalting was performed across Sephadex G-25 columns. CYSNO was synthesized in 0.5 N HCl, 0.5 mM EDTA and then neutralized (1:1) in 1 M phosphate buffer containing 0.5 mM EDTA. SNO-Hb(FeIII) preparations

25 followed a similar protocol, but used Hb(FeIII) as starting material. The latter was synthesized by treatment of Hb(FeII)O_2 with excess ferricyanide, followed by desalting across G-25 columns. SNO-Hb concentrations were verified spectroscopically and the S-nitrosothiol content was

30 determined by the method of Saville (Stamler, J.S., et al., *Proc. Nat. Acad. Sci USA* 89:444-448 (1992)). The S-NO/tetramer stoichiometry for both SNO-Hb preparations was

~2. Oxidation of the heme was undetectable by uv-spectrophotometric methods.

B. Potentialiation of SNO-Hb effects by Glutathione

Addition of glutathione (100 μ M) to bioassay chambers
5 potentiates the dose-response to both SNO-Hb(FeII)O₂ (■) and
SNO-Hb(FeIII) (●) (See Figure 4B. n = 6-12; p < 0.0001 for
both by ANOVA, compared with the respective tracings in
Figure 4A. Glutathione had a transient affect on baseline
tone in some experiments, and did not significantly
10 influence the response to Hb(FeII)O₂ (▲).

C. Transnitrosation between SNO-Hb and Glutathione

Rates of NO group transfer from SNO-Hb (100 μ M) to
glutathione (10 mM) are displayed for SNO-Hb(FeII)O₂ (oxy)
and SNO-Hb(FeIII) (met) (n=5). Data are presented as the
15 amount of GSNO formed relative to the starting SNO-Hb
concentration. The transfer is more rapid for SNO-
Hb(FeIII) than SNO-Hb(FeII)O₂ (p<0.002 by ANOVA),
suggesting that the GSNO/SNO-Hb equilibrium is shifted
toward GSNO by formation of metHb.

20 Methods

Thiol/SNO-Hb exchange, forming GSNO, was verified
chemically (Stamler, J.S., et al., *Proc. Natl Acad. Sci.*
USA, 89:444-448 (1992)) following trichloroacetic acid
precipitation (n=5). These results were verified in
25 separate experiments by measuring the residual SNO-Hb
concentration, following separation of reaction mixtures
across G-25 columns.

D. Export of S-nitrosothiols by red blood cells

Human red blood cells containing SNO-Hb are shown to
30 export low molecular weight RSNOs over time. Hemolysis,
which ranged from 0-<0.5% over one hour and did not

correlate with rates of RSNO release, could account for only a trivial fraction of the measured extracellular RSNO.

Methods

Packed human red blood cells were obtained by
5 centrifugation, washed, and resuspended in phosphate
buffered saline containing 5 mM SNOCYS (0.5 mM EDTA, pH
7.4) for one hour. This results in a red cell preparation
containing SNO-Hb (FeII O_2 /FeIII mixture) with a
stoichiometry of 0.5 S-NO/tetramer. The red blood cells
10 were then washed repeatedly to remove residual CYSNO
(verified), and incubated in Krebs' solution (1:4). The
accumulation of extracellular RSNO was measured over time
by the method of Saville (Saville, B., *Analyst*, 83:670-672
(1958)). Hemolysis was determined by spectral analysis of
15 red blood cell supernatants following centrifugation.

Example 5: SNO-Hb Bioactivity In Vivo

Systemic administration of cell-free Hb results in
hypertensive responses which have been attributed to NO
scavenging by the heme (Vogel, W.M., et al., *Am. J.*
20 *Physiol.* 251:H413-H420 (1986); Olsen, S.B., et al.,
Circulation 93:329-332 (1996)). To determine if SNO-Hb is
free of this adverse affect, and to explore if *in vitro*
mechanisms of NO release extend to the *in vivo*
circumstance, we compared responses to Hb and SNO-Hb
25 infused as a bolus into the femoral vein of anesthetized
rats. As illustrated in Figure 5, Hb(FeII) O_2 (200 nmol/kg)
caused an increase in mean arterial pressure of 20 ± 3 mm
Hg (n = 4; P < 0.05). In contrast, SNO-Hb(FeII) O_2 did not
exhibit hypertensive effects and SNO-Hb(FeIII) elicited
30 hypotensive responses (Figure 5). Thus, the profiles of
these compounds *in vivo* closely resemble those seen *in*
vitro (Figure 4A). Moreover, to demonstrate that the
physiological responses of red cells are comparable to

those of cell-free Hb preparations, erythrocytes containing SNO-Hb were injected into the femoral vein of rats pretreated with L-NMMA (50 mg/kg) to deplete endogenous RSNOs. At levels of SNO-Hb comparable to those found in the normal rat (0.1-0.5 μ M), SNO-Hb containing red blood cells elicited hypotensive responses (8 ± 1 mm Hg; mean \pm SEM; n=9), whereas native (SNO-Hb depleted) red blood cells did not (P=0.001). These changes in mean blood pressure of ~10% are on the order of those that differentiate normotension from hypertension in man, and in the therapeutic range of some antihypertensive regimens. The effects of both Hb and SNO-Hb -- whether cell-free or contained within red cells -- were transient, suggesting that S-nitrosylation of Hb and metabolism of SNO-Hb may be occurring *in vivo*, with consequent restoration of blood pressure. The bioactivity of SNO-Hb in blood, where S-NO/heme stoichiometries approach 1:50,000, is a dramatic illustration of the resistance of this NO-related activity to Hb(Fe) inactivation.

20 *In vivo* effects of cell-free Hb and SNO-Hbs

Administration of 2-200 nmol/kg Hb(FeII)O₂ (as a bolus) into the femoral vein of a Sprague-Dawley rat is shown to increase mean arterial pressure in a dose-dependent manner. At 200 nmol/kg, mean arterial pressure increased by 25 mm Hg (20 ± 3 mm Hg; n = 4; P < 0.05). Elevations in blood pressure reversed within 10-15 minutes. SNO-Hb(FeII)O₂ infusions (over the same dose range) are shown to ameliorate Hb(FeII)O₂-induced hypertension without causing overt changes in blood pressure. A similar response was seen at higher doses. By comparison, SNO-Hb(FeIII) infusions caused a significant fall in mean arterial pressure (pre 108 ± 4 mm Hg; post 74 ± 6 mm Hg, n = 5; P < 0.05) at the highest dose (200 nmol/kg). Hypotensive responses tended to be transient with blood

pressure normalizing over 10 minutes. A fall in blood pressure was also seen with injection of erythrocytes containing SNO-Hb.

5 Methods

Rats were anesthetized by intraperitoneal injection of pentobarbital and the femoral arteries and veins accessed by local cut down. The artery was then cannulated and the blood pressure monitored continuously using a Viggo Spectramed pressure transducer attached to a Gould recorder. An IBM PC (DATA Q Cudas) was used for data acquisition.

Example 6: Loading of Red Blood Cells With S-Nitrosothiols

Incubation of rat erythrocytes with S-nitrosocysteine (equimolar to heme (5 mM); phosphate buffer pH 7.4, 25°C) leads to rapid formation of intracellular S-nitrosothiols. MetHb does not form rapidly. Separation of cell content across G-25 columns establishes the formation of intraerythrocytic low molecular weight S-nitrosothiol, e.g. S-nitrosoglutathione, (GSNO). By 2 minutes, one can achieve as much as millimolar GSNO.

Method for assay of RSNO

S-nitrosocysteine (5 mM) treated red blood cells are pelleted rapidly by centrifugation, washed three times, lysed in deionized water at 4°C, and the cytosolic fraction subjected to rapid desalting across G-25 columns. Intracellular RSNO is measured by the method of Saville and can be confirmed spectroscopically.

Effects on blood pressure from loaded red blood cells

Red blood cells treated with S-nitrosocysteine (to produce SNO-RBCs) and introduced into the femoral vein of a Sprague-Dawley rat decreased mean arterial pressure in a

dose-dependent manner. For red blood cells in which SNO-Hb was assayed at 0.3 μ M (the endogenous in vivo SNO-Hb concentration), arterial pressure decreased by 8 ± 1 mm Hg (mean \pm SEM for 9 experiments; $p < 0.001$ compared to untreated red blood cell controls). For red blood cells in which SNO-Hb was assayed at 0.5 μ M, arterial pressure decreased by 10 mm Hg. For red blood cells in which SNO-Hb was assayed at 0.1 μ M (a sub-endogenous SNO-Hb concentration), arterial pressure decreased by 6 mm Hg. The administration of untreated red blood cells caused no effect or a slight increase in arterial blood pressure. Administration of L-monomethyl-L-arginine (L-NMMA; 50 mg/kg) caused an increase in blood pressure of about 20 mm Hg. Changes in blood pressure from a bolus administration of loaded red blood cells lasted 15-20 minutes.

Further methods

Rats were anesthetized by intraperitoneal injection of pentobarbital and the femoral arteries and veins accessed by local cut down. The artery was then cannulated and the blood pressure monitored continuously using a Viggo Spectramed pressure transducer attached to a Gould recorder. An IBM PC (DATA Q Cudas) was used for data acquisition.

Example 7: Effects of SNO-Hb on Coronary Vasodilation, Coronary Flow and Blood Pressure

SNO-Hb was synthesized as described in Example 4A. Completion of the reaction was determined as described in Example 4A. Twenty-four healthy mongrel dogs (25-30 kg) were anesthetized with intravenous thiamylal sodium (60-80 mg/kg) and subjected to left thoracotomy in the fourth intercostal space. The left circumflex coronary artery distal to the left atrial appendage was minimally dissected. A pair of 7-MHz piezoelectric crystals (1.5 X

2.5 mm, 15-20 mg) was attached to a Dacron backing and sutured to the adventitia on opposite surfaces of the dissected vessel segment with 6-0 prolene. Oscilloscope monitoring and on-line sonomicrometry (sonomicrometer 120-
5 2, Triton Technology, San Diego, CA) were used to ensure proper crystal position. A pulse Doppler flow probe (10 MHz, cuff type) was implanted distal to the crystals. An inflatable balloon occluder was also placed distal to the flow probe. All branches of the circumflex artery between
10 the crystals and the occluder were ligated. Heparin sodium-filled polyvinyl catheters were inserted into the left ventricular cavity via the apex, into the left atrium via the atrial appendage, and into the ascending aorta via the left internal thoracic artery. The catheters, tubing,
15 and wires were tunnelled to a subcutaneous pouch at the base of the neck.

After a 10 to 15 day recovery period, the catheters and wires were exteriorized under general anesthesia, and 2-3 days later, each dog was given a bolus injection of
20 SNO-Hb (0.4 mg) to evaluate vascular response. Two dogs that demonstrated <5% dilation of epicardial coronary vessels were excluded from subsequent studies, and two were excluded because of other technical reasons.

Dogs were trained and studied while loosely restrained
25 and lying awake in the lateral recumbent position. The laboratory was kept dimly illuminated and quiet. Aortic pressure, left ventricular end-diastolic pressure dp/dt external coronary diameter and coronary flow were monitored continuously. In 10 dogs, 0.1 ml of SNO-Hb solution, 50
30 nM/kg, was injected via the left atrial catheter. To verify potential effects of solvent on vasculature, 0.1 ml injections of 30% ethanol in distilled water were given as vehicle control. Between injections, phasic coronary blood flow and coronary artery diameter were allowed to return to
35 preinjection levels (minimum 15 minutes). Allowing a 15

minute period between injections resulted in no modification of repeated doses injections. To assess the direct and potential flow mediated indirect vasodilation effects of SNO-Hb on the conductance vessels, the dose was
5 repeated in 6 of 10 dogs with partial inflation of the adjustable occluder to maintain coronary blood flow at or slightly below preinjection levels. The response to acetylcholine chloride (Sigma Chemical) was assessed in another group of 10 dogs following a similar protocol to
10 that used for SNO-Hb.

Epicardial coronary diameter, coronary blood flow, heart rate, and aortic and left ventricular end-diagnostic pressures were compared before and after each SNO-Hb injection. The maximum changes in coronary dimension and
15 blood flow were expressed as a function of increasing doses of SNO-Hb. The response of coronary dimension to increasing doses followed a characteristic sigmoid dose-response curve that could be described by the following equation

$$Effect = \frac{maximal\ effect \times dose}{K_D + dose}$$

20 where K_D is the drug-receptor complex dissociation constant and is the dose at which 50% of the maximum response (EC_{50}) is achieved. In each animal, a nonlinear least-squares regression ($r^2 > 0.90$) was performed on the dose-response data. The regression was constrained to the above
25 equation. From the regression, values for maximum response and K_D were obtained for each individual animal. The mean of these values was then calculated to obtain an average K_D and maximum response for the study group. These values were used to generate a mean curve, which was plotted with
30 the mean dose-response values. (See Figures 6A-6F.)

Example 8: Endogenous Levels of S-nitrosohemoglobin and Nitrosyl(FeII)-Hemoglobin in Blood

To determine if SNO-Hb is naturally occurring in the blood, and if so, its relationship to the O₂ transport capacity and nitrosylated-heme content of red cells, an analytical approach was developed to assay the S-nitrosothiol and nitrosyl-heme content of erythrocytes (Table 2). Arterial blood was obtained from the left ventricle of anesthetized rats by direct puncture and venous blood was obtained from the jugular vein and inferior vena cava. Hb was then purified from red cells and assayed for RSN-O and (FeII)NO content. Arterial blood contained significant levels of SNO-Hb, whereas levels were virtually undetectable in venous blood (Table 2).

Measurements made 45 minutes after infusion of the NO synthase inhibitor N^ω-monomethyl-L-arginine (L-NMMA) (50 mg/kg), showed a depletion of SNO-Hb as well as total Hb-NO (82 and 50 ± 18%, respectively; n = 3-5; p < 0.05). These data establish the endogenous origin of SNO-Hb, although some environmental contribution is not excluded. The arterial-venous distribution seen for SNO-Hb was reversed in the case of Hb(FeII)NO, which was detected in higher concentrations in partially deoxygenated (venous) erythrocytes (Table 2). Accordingly, the proportion of nitrosylated protein thiol and heme appears to depend on the oxygenation state of the blood. Consistent with these findings, Wennmalm and coworkers have shown that Hb(FeII)NO forms mainly in venous (partially deoxygenated) blood (Wennmalm, A., et al., *Br. J. Pharmacol.* 106(3):507-508 (1992)). However, levels of Hb(FeII)NO in vivo are typically too low to be detected (by EPR) and SNO-Hb is EPR-silent (i.e., it is not paramagnetic). Thus, photolysis-chemiluminescence represents an important technological advance, as it is the first methodology

capable of making quantitative and functional assessments of NO binding to Hb under normal physiological conditions.

Table 2

Endogenous levels of S-nitrosohemoglobin and nitrosyl(FeII)-hemoglobin in blood

Site	SNO-Hb (nM)	Hb(FeII)NO (nM)
Arterial	311 ± 55*	536 ± 99 †
Venous	32 ± 14	894 ± 126

* P < 0.05 vs venous; † P < 0.05 for paired samples vs venous

Methods

Blood was obtained from the left ventricle (arterial) and jugular vein (venous) of anesthetized Sprague-Dawley rats. Comparable venous values were obtained in blood from the inferior vena cava. Red blood cells were isolated by centrifugation at 800 g, washed three times in phosphate buffered saline at 4°C, lysed by the addition of 4-fold excess volume of deionized water containing 0.5 mM EDTA, and desalted rapidly across G-25 columns according to the method of Penefsky at 4°C. In 24 rats, Hb samples were divided in two aliquots which were then treated or not treated with 10-fold excess HgCl₂ over protein concentration as measured by the method of Bradford. Determinations of SNO-Hb and Hb(FeII)NO were made by photolysis-chemiluminescence as described below. In 12 additional rats, further verification of the presence of SNO-Hb was made by assaying for nitrite after HgCl₂ treatment. Specifically, samples (with and without HgCl₂) were separated across Amicon-3 (Centricon filters, m.w. cut off 3,000) at 4°C for 1 h, and the low molecular weight fractions collected in airtight syringes containing 1 μM

glutathione in 0.5 N HCl. Under these conditions, any nitrite present was converted to S-nitrosoglutathione, which was then measured by photolysis-chemiluminescence (detection limit ~ 1 nM). SNO-Hb was present in all
5 arterial samples, and levels determined by this method (286 ± 33 nM) were virtually identical to and not statistically different from those shown in Table 2. In venous blood, SNO-Hb was undetectable (0.00 ± 25 nM); levels were not statistically different from those given above.

10 Method for assay of S-nitrosohemoglobin

A highly sensitive photolysis-chemiluminescence methodology was employed. A somewhat similar assay has been used for measuring RSNOs (S-nitrosothiols) in biological systems (Gaston, B., et al., *Proc. Natl. Acad. Sci. USA* 90:10957-10961 (1993); Stamler, J.S., et al.,
15 *Proc. Natl. Acad. Sci. USA* 89:7675-7677 (1992)). The method involves photolytic liberation of NO from the thiol, which is then detected in a chemiluminescence spectrometer by reaction with ozone. The same principle of operation
20 can be used to cleave (and measure) NO from nitrosyl-metal compounds (Antonini, E. and Brunori, M. In *Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York, pp. 29-31 (1971)). With adjustment of flow rates in the photolysis cell,
25 complete photolysis of the NO ligand of Hb(FeII)NO could be achieved. Standard curves derived from synthetic preparations of SNO-Hb, Hb(FeII)NO, and S-nitrosoglutathione were linear ($R > 0.99$), virtually superimposable, and revealing of sensitivity limits of
30 approximately 1 nM. Two analytical criteria were then found to reliably distinguish SNO-Hb from Hb(FeII)NO: 1) signals from SNO-Hb were eliminated by pretreatment of samples with 10-fold excess $HgCl_2$, while Hb(FeII)NO was resistant to mercury challenge; and 2) treatment of SNO-Hb

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with HgCl_2 produced nitrite (by standard Griess reactions) in quantitative yields, whereas similar treatment of Hb(FeII)NO did not. UV/VIS spectroscopy confirmed that NO remained attached to heme in the presence of excess HgCl_2 .

5 Example 9: Inhibition of Platelet Aggregation by
S-Nitrosohemoglobins

Methods to prepare human HbA_0 were as described in Example 1 "Methods" section. Methods to make SNO-Hb(FeII)O_2 were as described for Example 2A. Methods to
10 make SNO-Hb(FeIII) were as in Example 1 (see parts B, C, and "Methods" in Example 1). Quantifications of SNO-hemoglobins were made as in Example 1 according to the method of Saville (Saville, B., *Analyst* 83:670-672 (1958)) and by the assay as described in Example 8, "Method for
15 assay of S-nitrosohemoglobin."

Venous blood, anticoagulated with 3.4 mM sodium citrate, was obtained from volunteers who had not consumed acetylsalicylic acid or any other platelet-active agent for at least 10 days. Platelet-rich plasma was prepared by
20 centrifugation at $150 \times g$ for 10 minutes at 25°C and was used within 2 hours of collection. Platelet counts were determined with a Coulter counter (model ZM) to be 1.5 to $3 \times 10^8/\text{ml}$.

Aggregation of platelet-rich plasma was monitored by a
25 standard nephelometric technique in which results have been shown to correlate with bleeding times. Aliquots (0.3 ml) of platelets were incubated at 37°C and stirred at 1000 rpm in a PAP-4 aggregometer (Biodata, Hatsboro, PA). Hemoglobins were preincubated with platelets for 10 min and
30 aggregations were induced with $5 \mu\text{M}$ ADP. Aggregations were quantified by measuring the maximal rate and extent of change of light transmittance and are expressed as a normalized value relative to control aggregations performed in the absence of hemoglobin.

The results of the aggregation assays are shown in Figures 7A, 7B and 7C. Standard deviations are shown as vertical bars. SNO-Hb[Fe(II)]O₂ causes some inhibition of platelet aggregation at the higher concentrations tested.

- 5 SNO-Hb[Fe(III)] also inhibits platelet aggregation when present at concentrations of 1 μ M and above, but to a much greater extent than SNO-Hb[Fe(II)]O₂.

Example 10: Effect of SNO-Hbs on cGMP

- Platelet rich plasma (PRP) was incubated with either
10 hemoglobin, SNO-oxy Hb, or SNO-metHb for 5 min, after which the assay was terminated by the addition of 0.5 ml of ice cold trichloroacetic acid to 10%. Ether extractions of the supernatant were performed to remove trichloroacetic acid, and acetylation of samples with acetic anhydride was used
15 to increase the sensitivity of the assay. Measurements of cyclic GMP were performed by radioimmunoassay (Stamler, J. et al., *Circ. Res.* 65:789-795 (1989)).

- Results are shown in Figure 8. For all concentrations of Hb tested (1, 10 and 100 μ M), the concentration of cGMP
20 measured for SNO-Hb(FeIII) was less than that of native Hb.

Example 11: Polynitrosation of Hb

- A. HbA₀ (oxy) was incubated with S-nitrosoglutathione at a ratio of 6.25 S-nitrosoglutathione/HbA₀ for 240 minutes at pH 7.4 at 25°C and desalted over Sephadex G-25 columns.
25 Spectra were run in the presence (spectrum B, Figure 9A) and absence (spectrum A, Figure 9A) of dithionite. The shift in the spectrum is indicative of 2 SNO groups/tetramer.

- B. HbA₀ was incubated with 100-fold excess S-nitrosoglutathione over protein for 240 minutes at pH 9.2,
30 followed by desalting over a G-25 column. A portion was

then treated with dithionite. The spectra in Figure 9B indicate that Hb has been nitrosated at multiple sites.

C. HbA₀ was treated with 100-fold excess S-nitrosocysteine over tetramer at pH 7.4, 25°C for 5-20 min. After various times of treatment, the protein was desalted over a G-25 column and treated with dithionite. The spectra show progressive polynitrosation of Hb with time (spectra A to F in Figure 9C). After 5 minutes of treatment with 100-fold excess S-nitrosocysteine, 0.09 NO groups had added per tetramer (spectrum A of Figure 9C); after 20 minutes, at least 4 NO groups had added (spectrum F). At intermediate time points, 0.4 NO groups (spectrum B), 1.58 NOs (spectrum C), 2.75 NOs (spectrum D) or 2.82 NOs had added per tetramer (spectrum E).

D. Rat Hb was treated with 100x S-nitrosoglutathione excess over tetramer for 3 hours at pH 7.4. The protein was then desalted by passage through a G-25 column. A portion of the desalted protein was treated with dithionite (spectrum B in Figure 9D; the protein of spectrum A was left untreated by dithionite). Spectrum B in Figure 9D is illustrative of a ratio of 6 RNOs/Hb.

E. A time course experiment tracking the extent of nitrosation of HbA₀ with time was performed (Figure 9E). Treatment of HbA₀ was with 10x excess S-nitrosocysteine at pH 7.4, 25°C or with 100x excess S-nitrosocysteine under the same conditions. Analysis for SNO and NO was performed by the method of Saville and by UV spectroscopy as in Jia, L. et al., *Nature* 380:221-226 (1996). Under these conditions the heme is ultimately oxidized; the rate is time dependent.

Treatment with 10x excess S-nitrosocysteine nitrosylates only the thiol groups of the two reactive

cysteine residues of HbA₀. Inositol hexaphosphate is known to shift the allosteric equilibrium towards the T-structure (ordinarily, the deoxy form). Treatment with 100x excess nitrosates additional groups; i.e., the product has more
5 than 2 NO groups/tetramer.

Example 12: Effect of SNO-Hb(FeII)O₂ on Blood Flow

SNO-Hb(FeII)O₂, having a SNO/Hb ratio of 2, was prepared (from HbA₀) by reaction with S-nitrosothiol. Rats breathing 21% O₂ were injected (time 0) with Hbs prepared
10 from HbA₀ as indicated in Figure 10 (open circles, SNO-Hb (100 nmol/kg); filled circles, SNO-Hb (1000 nmol/kg); filled squares, unmodified Hb (1000 nmol/kg)). Three rats were used per experiment. Blood flow was measured in brain using the H₂ clearance method; microelectrodes were placed
15 in the brain stereotactically. Concomitant PO₂ measurements revealed tissue PO₂ = 20. Thus, SNO-Hb improves blood flow to the brain under normal physiological conditions, whereas native Hb decreases blood flow. NO group release is promoted by local tissue hypoxia.

20 Example 13: Effects of SNO-Hb(FeII)O₂, SNO-Hb(FeIII) and (NO)_x-Hb(FeIII) on Tension of Rabbit Aorta

Hemoglobin was treated with either 1:1, 10:1 or 100:1 S-nitrosocysteine to Hb tetramer for 1 hour, processed as in Example 4. The products of the reactions done with 1:1
25 and 10:1 excess were assayed by the Saville assay and by standard spectrophotometric methods. The product of the reaction done at the 1:1 ratio is SNO-Hb(Fe)O₂; SNO-Hb(FeIII) is found following reaction with 1:10 excess CYSNO/tetramer.

30 The aortic ring bioassay was performed as described in Example 4. The product of the reaction in which a ratio of 100:1 CYSNO/Hb tetramer was used, contains 2 SNOs as well as NO attached to the heme. The potency of the 100:1

CYSNO/Hb product is much greater than that of SNO-Hb(FeIII) and is indicative of polynitrosation (see Figure 11).

Example 14: Effect of Oxygenation on Partially Nitrosylated Hemoglobin

5 The effect of oxygenation on partially nitrosylated Hb was examined by following spectral changes in the Soret region upon the addition of air to partially nitrosylated Hb. Hemoglobin A (17 μ M) was deoxygenated by bubbling argon through a 1 ml solution in 100 mM phosphate (pH 7.4),
10 for 45 minutes. Nitric oxide was added by injection of 0.5 μ l of a 2 mM solution, stored under nitrogen. The final heme:NO ratio was 68:1. The solution was slowly aerated by sequential 50 μ l injections of room air. Figure 12 shows that the initial additions of air failed to produce a true
15 isobestic point, indicating changes in the concentrations of at least three absorbent species. Later additions of air did produce a true isobestic point, indicative of the conversion of deoxyhemoglobin to oxyhemoglobin, with the loss of nitrosyl heme. The results show that nitrosylated
20 Hb is not a stable end product.

Example 15: Conversion of Nitrosylhemoglobin to SNO-Hemoglobin

 The hypothesis that the nitric oxide was being transferred from the heme iron to a thiol residue, forming
25 nitrosothiol upon oxygenation, was tested. Hemoglobin A (400 μ M) was deoxygenated by bubbling argon through a 1 ml solution in 100 mM phosphate (pH 7.4), for 45 minutes. Nitric oxide was added by injection of an appropriate volume of a 2 mM solution, stored under nitrogen, to
30 achieve different NO/Hb ratios. The solutions were then exposed to air by vigorous vortexing in an open container. Samples were then analyzed by Saville assay and by chemiluminescence after UV photolysis. Data are shown as

mean \pm standard error ($n > 3$). Figure 13 shows that S-nitrosothiol is formed in this manner, and that the efficiency of this reaction is greatest at high ratios of heme to nitric oxide. Amounts are highest at very high NO/Hb ratios, i.e., $> 2:1$. This result implies that nitrosyl Hb entering the lung is converted into SNO-Hb, as under physiological conditions the ratio of heme to NO is high.

Example 16: Effects Dependent upon Heme:NO Ratio

10 It was proposed that the binding of nitric oxide to the heme of the β chain was inherently unstable, and that the reason for lower yields of SNO-Hb at higher concentrations of nitric oxide, was a loss of bound nitric oxide as a result of this instability. Hemoglobin A ($17.5 \mu\text{M}$) was
15 deoxygenated by bubbling argon through a 1 ml solution in 100 mM phosphate (pH 7.4), for 45 minutes. Nitric oxide was added by sequential injections of an appropriate volume of a 2 mM solution, stored under nitrogen. Figure 14A: Difference spectra of the nitric oxide hemoglobin mixture
20 and the starting deoxyhemoglobin spectrum are shown. Figure 14B: The peak wavelength of the difference spectra plotted against the concentration of nitric oxide added to the solution. These data show that addition of small amounts of nitric oxide (heme:NO ratios of approximately
25 70:1) produce predominantly nitrosylhemoglobin and some oxidized hemoglobin. However, nitric oxide additions of the order of $10 \mu\text{M}$ result in the formation of oxidized hemoglobin. Heme:NO ratios at this point are approximately 7:1. As the concentration of nitric oxide is increased by
30 further additions of nitric oxide, the predominant species formed becomes nitrosylhemoglobin (heme:NO ratio 1:1). The results in Figures 14A and 14B show that under anaerobic conditions, the addition of increasing quantities of nitric oxide to Hb results first in the production of

nitrosylhemoglobin and then oxidized Hb (metHb). At very high levels of nitric oxide, nitrosyl-hemoglobin is once again seen as the nitric oxide first reduces metHb to deoxyHb (producing nitrite), then binds NO. This drives the conformational change of T-structure Hb to R-structure, stabilizing the β heme-nitric oxide bond. The appearance of oxidized Hb at heme to nitric oxide ratios of approximately 10:1 indicates the decay of the heme/NO bond to produce oxidized Hb and nitric oxide anion (nitroxyl). The presence of nitric oxide anion was confirmed by detection of N_2O in the gas phase by gas chromatography mass spectrometry and by the production of NH_2OH .

Example 17: Effects upon Oxygenation of Nitrosyl-deoxyHb

Hemoglobin A ($20.0 \mu M$) was deoxygenated by bubbling argon through a 1 ml solution in 100 mM phosphate (pH 7.4), for 45 minutes. In both Figure 15A and Figure 15B, the lowest to the highest spectra indicate the sequential additions of air. These are difference spectra in which the pure deoxyHb spectrum occurs at zero absorbance. The peak at 419 nm is from nitrosylhemoglobin; oxidized hemoglobin absorbs at 405 nm.

In the experiments shown in Figure 15A, hemoglobin was gradually oxygenated by sequential additions of $10 \mu l$ of room air by Hamilton syringe. Spectra are shown as difference spectra from the initial deoxyhemoglobin spectrum. In the experiments shown in Figure 15B, nitric oxide ($1 \mu M$) was added by injection of $0.5 \mu l$ of a 2 mM solution, stored under nitrogen. Final heme:NO ratio was 80:1. The solution was gradually oxygenated by sequential additions of $10 \mu l$ of room air. Spectra are shown as difference spectra from the initial deoxyhemoglobin spectrum. These data show the initial formation of a nitrosylhemoglobin peak, along with some formation of oxidized hemoglobin, which disappears after the addition of

approximately 30 μ l of air. The results indicate that a small quantity of nitrosyl Hb is formed upon addition of low ratios of nitric oxide to deoxy Hb, and that this nitrosyl Hb is lost upon oxygenation.

5 Example 18: Role of β 93Cys in Destabilizing Nitrosyl-Heme

Recombinant hemoglobins were obtained from Clara Fonticelli at the University of Maryland School of Medicine. β 93Ala represents a single amino acid substitution within human hemoglobin A, whilst β 93Cys
10 represents a wild type control. Recombinant hemoglobin (5 μ M containing either a wild type cysteine (β 93Cys) or a mutant alanine (β 93Ala) at position β 93 was deoxygenated as in Figures 15A and 15B. Nitric oxide (1 μ M) was added by injection of 0.5 μ l of a 2 mM solution, stored under
15 nitrogen. The final heme:NO ratio was 20:1. The solution was gradually oxygenated by sequential additions of 10 μ l of room air. The absorption at 418 nm of difference spectra versus initial deoxyhemoglobin spectra is shown in Figure 16. These data indicate that within the mutant, a
20 nitrosyl adduct was formed that was not lost upon addition of room air. However, the nitrosyl adduct formed within the wild type was lost after addition of greater than 10 μ l of room air. This shows that NO is not lost from this nitrosyl (FeII) heme in a mutant Hb that does not possess a
25 thiol residue at position β 93. Therefore, this thiol, which is in close proximity to the heme within the R-structure, is critical for destabilizing the heme nitric oxide bond.

Example 19: SNO-Hb from Nitrosyl-Hb Driven by O₂

30 Hemoglobin A (400 μ M) was prepared in a 1 ml solution, in 100 mM phosphate (pH 7.4). Nitric oxide was added by injection of an appropriate volume of a 2 mM solution, stored under nitrogen. The solutions were vortexed

vigorously in an open container. Samples were then analyzed by Saville assay and by chemiluminescence after UV photolysis. The results in Figure 17 show that S-nitrosothiol Hb can be formed from oxyHb, but that the efficiency of this formation is critically dependent upon the ratio of heme to nitric oxide.

Example 20: Formation of Oxidized Hb Dependent on Protein Concentration

Hemoglobin A was diluted to the concentrations indicated by the different symbols in Figure 18A and Figure 18B, in 50 ml of 100 mM phosphate buffer (pH 7.4). Nitric oxide was added by sequential injections of an appropriate volume of a 2 mM solution, stored under nitrogen. After each injection, the absorbance at 415 and 405 nm was measured. The ratio of these two absorbances was used to calculate the percentage content of oxidized hemoglobin (Figure 18A), and the absolute yield of oxidized hemoglobin (Figure 18B). ♦ represents 1.26 μ M hemoglobin, ■ represents 5.6 μ M hemoglobin, ▲ represents 7.0 μ M hemoglobin, X represents 10.3 μ M hemoglobin, ✕ represents 13.3 μ M hemoglobin, and ● represents 18.3 μ M hemoglobin. These data show that only a small proportion of the nitric oxide added results in the formation of oxidized hemoglobin (<10%). Furthermore, this tendency to form oxidized hemoglobin is reduced at higher protein concentrations.

Example 21: Effect of Ionic Strength and NO:Hb Ratio on Extent of MetHb Formation

We proposed that the degree of hydrogen bonding between bound oxygen and the distal histidine was critical in determining the degree of oxidation of hemoglobin by nitric oxide. Therefore, we examined the degree of oxidation of hemoglobin by nitric oxide in a variety of buffers. 5 ml of phosphate buffer containing 300 μ M Hemoglobin A (~95%

oxyHb) was placed in a 15 ml vial. Nitric oxide was added from a stock solution, 2 mM, stored under nitrogen.

Immediately after nitric oxide addition, the absorbance at 630 nm was measured, and the concentration of oxidized

5 (metHb) was plotted, using 4.4 as the extinction coefficient for metHb at 630 nm. Experiments were performed in 1 M, 100 mM, and 10 mM sodium phosphate buffer (pH 7.4). The data in Figure 19 show higher oxidized hemoglobin formation in 1M phosphate, which is indicative
10 of a higher effective substrate concentration, as would be predicted by phosphate destabilization of the hydrogen bond between iron bound oxygen and the distal histidine. At the lowest concentrations of nitric oxide added, S-nitrosothiol was formed under all conditions (approximately 5 μ M).
15 Additions of nitric oxide at concentrations of 30 μ M or greater resulted in the additional formation of nitrite. The presence of 200 mM borate within the buffer reduced oxidized hemoglobin and nitrite formation, whilst the presence of either 200 mM or chloride increased the
20 formation of oxidized hemoglobin and nitrite. Addition of nitric oxide to hemoglobin in 10 mM phosphate buffer at a ratio of less than 1:30 (NO:Hemoglobin A) resulted in the formation of S-nitrosothiol without production of oxidized hemoglobin. S-nitrosothiol formation was optimized by
25 adding the nitric oxide to hemoglobin in 10 mM phosphate, 200 mM borate, pH 7.4. Therefore, the balance between oxidation and nitrosothiol formation is dependent upon the ratio of nitric oxide to hemoglobin and the buffer environment.

30 Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention

CLAIMS

What is claimed is:

1. A method for delivering NO to cells in a mammal,
comprising administering low molecular weight
5 nitrosating agent to the mammal.
2. A method for increasing O₂-delivery capacity of
hemoglobin in a mammal, comprising administering a low
molecular weight NO donating agent to the mammal.
3. A method for scavenging oxygen free radicals in a
10 mammal, comprising administering low molecular weight
nitrosating agent to the mammal.
4. A method for preserving a living organ ex vivo,
comprising perfusing the organ with a composition
comprising nitrosated hemoglobin and low molecular
15 weight thiol or NO donating agent.
5. A method for treating a blood borne disease in a
patient, comprising the steps of:
 - a) isolating the patient's red blood cells;
 - b) treating the patient's red blood cells with S-
20 nitrosothiol; and
 - c) readministering to the patient the red blood
cells.
6. The method of Claim 5, wherein the blood borne disease
is malaria.
- 25 7. A method for treating a disease or medical disorder in
a mammal, comprising administering to the mammal a
nitrosating agent.

8. The method of Claim 7 in which the nitrosating agent is selected for rapid entry into the target cell.
9. The method of Claim 7, wherein the disease or medical disorder is selected from the group consisting of:
5 shock, angina, stroke, reperfusion injury, acute lung injury, sickle cell anemia and infection of red blood cells.
10. A composition comprising SNO-Hb[FeII]O₂ which is S-nitrosylated without detectable oxidation of the heme Fe.
10
11. A method for making SNO-Hb[FeII]O₂, which is specifically S-nitrosylated on thiol groups, comprising incubating excess nitrosating agent with purified hemoglobin in the presence of oxygen.
- 15 12. The method of Claim 11 in which the nitrosating agent is a low molecular weight S-nitrosothiol.
13. A composition comprising SNO-Hb[FeII] which is S-nitrosylated without detectable oxidation of the heme Fe.
- 20 14. A method for making SNO-Hb[FeII], which is specifically S-nitrosylated on thiol groups, comprising incubating excess nitrosating agent with purified hemoglobin in the absence of oxygen.
15. The method of Claim 14 in which the nitrosating agent
25 is a low molecular weight S-nitrosothiol.
16. A method for regulating delivery of oxygen and NO, in various redox forms, in a mammal, comprising

administering to the mammal a mixture of a low molecular weight thiol or nitrosothiol and hemoglobin or nitrosated hemoglobin, selected for the oxidation state of the heme iron and for the oxygenation state.

- 5 17. A method for delivering NO in a mammal, comprising administering to the mammal a blood substitute comprising nitrosated hemoglobin.
18. The method of Claim 17, in which the blood substitute comprises nitrosated hemoglobin and low molecular
10 weight S-nitrosothiol.
19. A method for scavenging oxygen free radicals and NO \cdot in a mammal, comprising administering to the mammal a blood substitute comprising nitrosated hemoglobin.
- 15 20. A method for reducing blood pressure in a mammal, comprising administering nitrosated hemoglobin to the mammal.
21. A method for treating a disease in a mammal, comprising administering a form of nitrosated or nitrated
20 hemoglobin to the mammal, wherein the disease is selected from the group consisting of heart disease, brain disease, vascular disease, atherosclerosis, lung disease and inflammation.
22. A method for treating a medical condition in a mammal,
25 comprising administering a form of nitrosated hemoglobin to the mammal, wherein the medical condition is selected from the group consisting of stroke, angina and acute respiratory distress.

23. A method for enhancing the preservation of an excised organ, comprising storing the organ in a solution comprising SNO-Hb(FeII)O₂.
24. A method for treating a human with sickle cell anemia
5 comprising administering to the human a preparation comprising SNO-Hb(FeII)O₂.
25. The method of Claim 24 in which the preparation comprises SNO-Hb(FeII)O₂ and a thiol.
26. The method of Claim 24 in which the preparation
10 comprises SNO-Hb(FeII)O₂ and an S-nitrosothiol.
27. A method for treating a patient having a disease or medical condition characterized by abnormalities of nitric oxide and oxygen metabolism, comprising
15 administering to the patient an effective amount of a preparation comprising nitrosated hemoglobin.
28. The method of Claim 27 in which the disease or medical condition is selected from the group consisting of: heart disease, lung disease, sickle-cell anemia, stroke, sepsis or organ transplantation.
- 20 29. A blood substitute comprising nitrosated or nitrated hemoglobin.
30. A method for treating a disorder resulting from platelet activation or adherence in an animal or human, comprising administering a composition comprising
25 nitrosated or nitrated hemoglobin in a therapeutically effective amount.

31. The method of Claim 30 wherein the disorder is selected from the group consisting of: myocardial infarction, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis, sepsis and unstable angina.
- 5 32. A method for preventing thrombus formation in an animal or human, comprising administering a composition comprising nitrosated hemoglobin in a therapeutically effective amount.
- 10 33. A method for regulating platelet activation in an animal or human, comprising administering, in a therapeutically effective amount, a composition comprising a substance which controls the allosteric equilibrium or spin state of hemoglobin.
- 15 34. The method of Claim 33 in which the substance converts the allosteric state of hemoglobin from R-structure to T-structure.
- 20 35. A method for forming polynitrosated hemoglobin, comprising combining hemoglobin with an excess of S-nitrosothiol over hemoglobin tetramer in an aqueous solution, and maintaining the resulting combination under conditions appropriate for nitrosation to occur at multiple sites on hemoglobin.
- 25 36. A method for forming polynitrosated or polynitrated hemoglobin in which heme Fe is in the FeII state, comprising combining hemoglobin with an NO donating compound, maintaining the resulting combination under conditions appropriate for nitrosation or nitration to occur, thereby forming polynitrosated or polynitrated hemoglobin, and reacting the polynitrosated or

polynitrated hemoglobin with a reagent which selectively reduces FeIII to FeII.

37. The method of Claim 36 in which the reagent which selectively reduces FeIII to FeII is a cyanoborhydride.
- 5 38. The method of Claim 36 in which the reagent which selectively reduces FeIII to FeII is methemoglobin reductase.
39. A composition comprising polynitrosated hemoglobin.
- 10 40. Method for treating or preventing a disease or medical disorder which can be ameliorated by delivery of NO or its biological equivalent to tissues affected by the disease or medical disorder, in an animal or human, comprising administering to the animal or human nitrosyl-heme-containing donors of NO.
- 15 41. The method of Claim 40 wherein the nitrosyl-heme-containing donor of NO is nitrosylhemoglobin.
- 20 42. Method for making stable nitrosyl-deoxyhemoglobin comprising adding NO to deoxyhemoglobin in an aqueous solution such that the ratio of NO:heme is less than about 1:100 or greater than about 0.75.
43. Method for making SNO-oxyhemoglobin, comprising adding NO to an aqueous solution of oxyhemoglobin and buffer having a pK of at least about 9.4, at a concentration of approximately 10 mM to 200 mM, at pH 7.4.
- 25 44. Method for making SNO-oxyhemoglobin, comprising adding NO to an aqueous solution of oxyhemoglobin in approximately 10 mM phosphate buffer at pH 7.4.

45. A composition comprising nitrosyl-deoxyhemoglobin in a physiologically compatible buffer, wherein the ratio of NO:heme is less than about 1:100 or greater than about 0.75.
- 5 46. Method for making nitrosyl-oxyhemoglobin comprising adding NO to oxyhemoglobin in an aqueous solution such that the ratio of NO:hemoglobin is less than about 1:30.
47. Hemoglobin conjugated to an NO-donor.
- 10 48. Hemoglobin of Claim 47, wherein the NO-donor is selected from the group consisting of:
diazoniumdiolates, nitroprusside, nitroglycerin and nitrosothiol.
- 15 49. A composition comprising hemoglobin and one or more NO-donors.
- 20 50. A method for treating or preventing a disease or medical disorder which can be ameliorated by delivery of NO or its biological equivalent to tissues affected by the disease or medical disorder, in an animal or human, comprising administering a heme-based blood substitute and inhaled NO to the animal or human.
51. A method for delivering CO to the tissues in an animal or human, comprising administering CO-derivatized hemoglobin to the animal or human.
- 25 52. A method of treating or preventing a disease or medical disorder which can be ameliorated by delivery of NO or its biological equivalent to tissues affected by the disease or medical disorder in an animal or human,

comprising administering both CO-derivatized hemoglobin and a nitrosated hemoglobin to the animal or human.

53. Nitrosylhemoglobin conjugated to one or more electron acceptors.
- 5 54. Nitrosylhemoglobin of Claim 53, wherein the electron acceptor is selected from the group consisting of: superoxide dismutase, stable nitroxide radicals, and oxidized forms of nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide, flavin mononucleotide, ascorbate and dehydroascorbate.
- 10
55. A composition comprising nitrosylhemoglobin and one or more electron acceptors.
56. Hemoglobin conjugated to nitric oxide synthase.
- 15 57. Hemoglobin of Claim 56, wherein the nitric oxide synthase is nitric oxide synthase of neurons.
58. A composition comprising hemoglobin and nitric oxide synthase.
59. A method for making erythrocytes comprising
- 20 nitrosylhemoglobin, comprising incubating deoxygenated erythrocytes in a solution comprising NO.
60. Erythrocytes comprising nitrosylhemoglobin.
61. A method for treating shock in an animal or human comprising administering hemoglobin α -chains to the
- 25 animal or human.

62. A method for treating or preventing a disease or
medical disorder which can be ameliorated by delivery
of NO or its biological equivalent to tissues affected
by the disease or medical disorder, in an animal or
5 human, comprising administering hemoglobin β -chains to
the animal or human.

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Diseases which can be ameliorated by delivery of NO to
5 tissues affected by the disease can be treated by
administration of nitrosyl-heme-containing donors of NO,
including nitrosylhemoglobin. Nitrosylhemoglobin can be
made by the reaction of NO with hemoglobin under certain
conditions in which the NO:hemoglobin ratio is critical,
10 and is converted to SNO-Hb under physiological conditions.

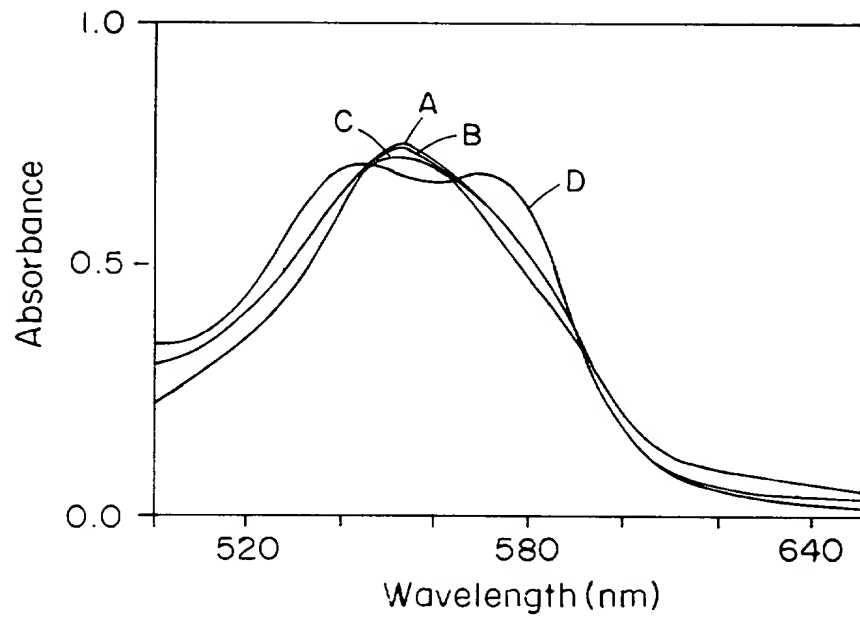


FIG. 1A

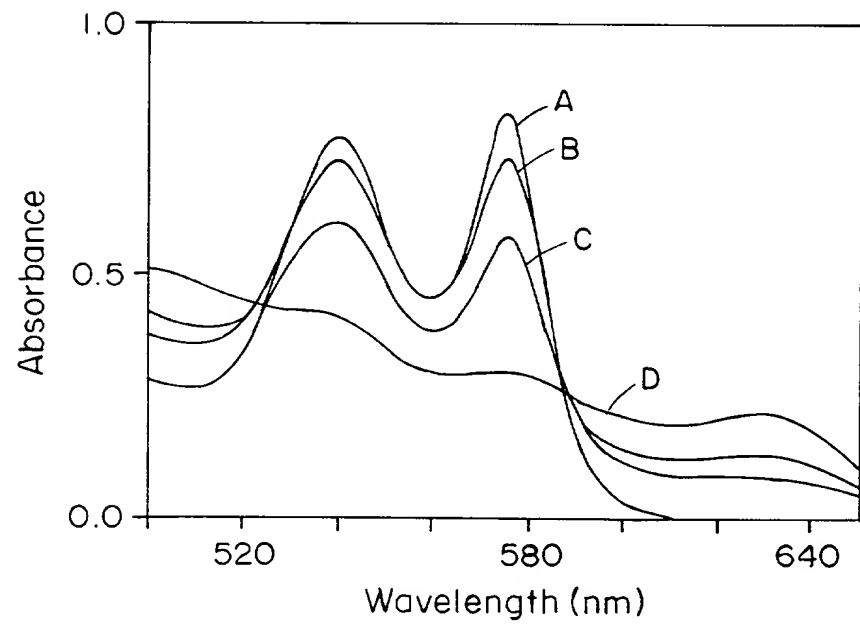


FIG. 1B

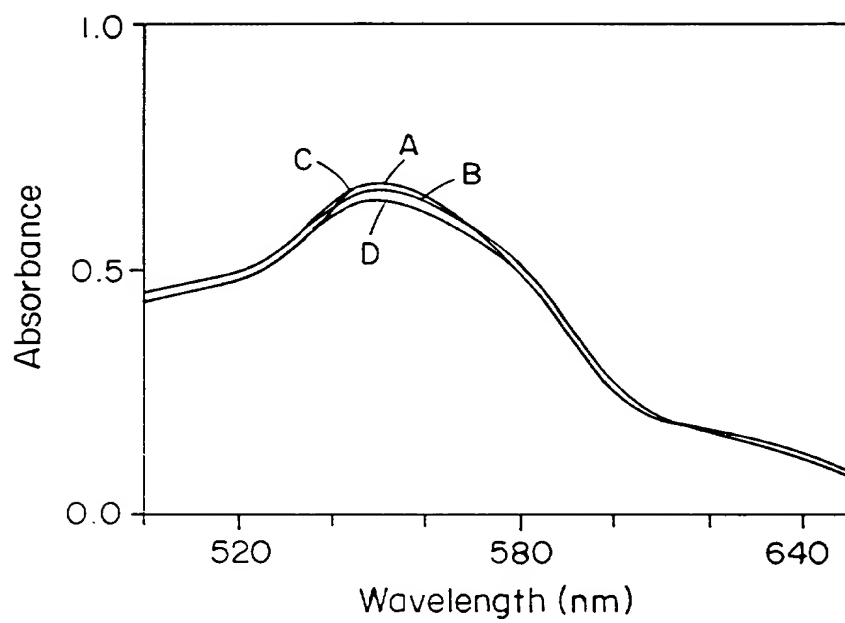


FIG. IC

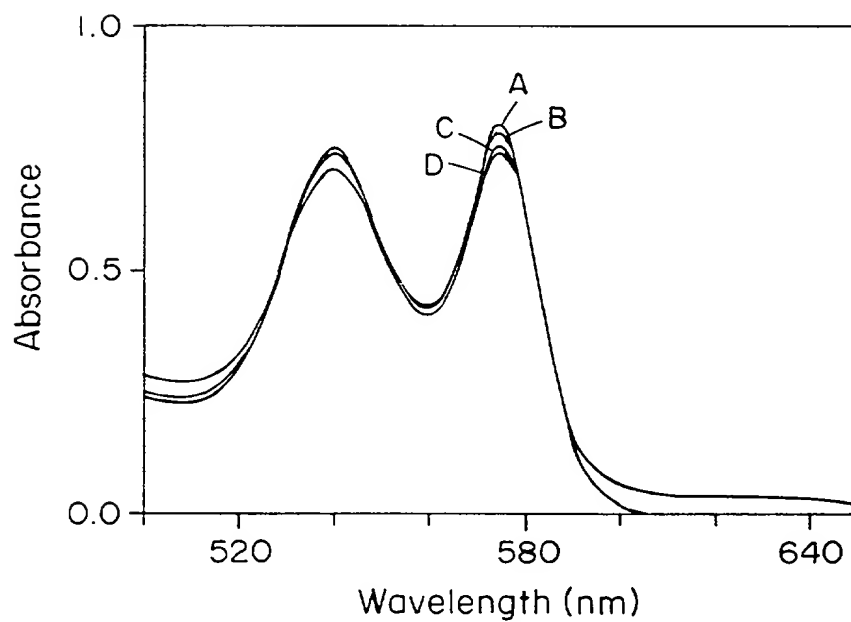


FIG. ID

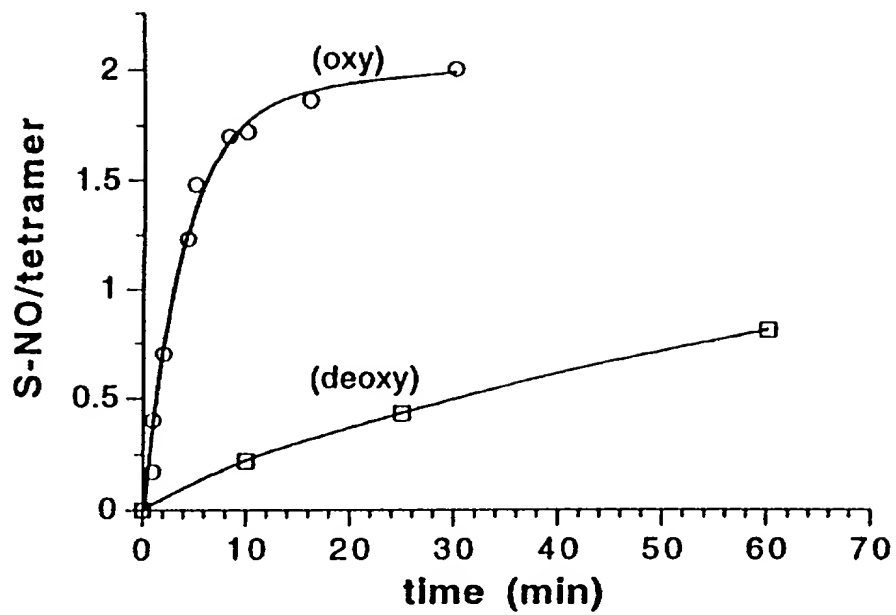


FIG. 2A

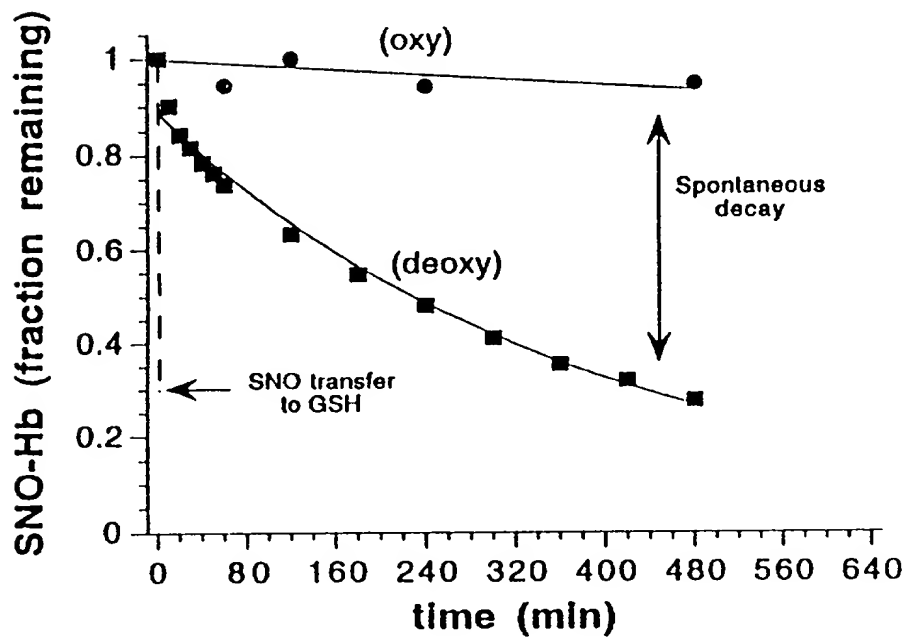


FIG. 2B

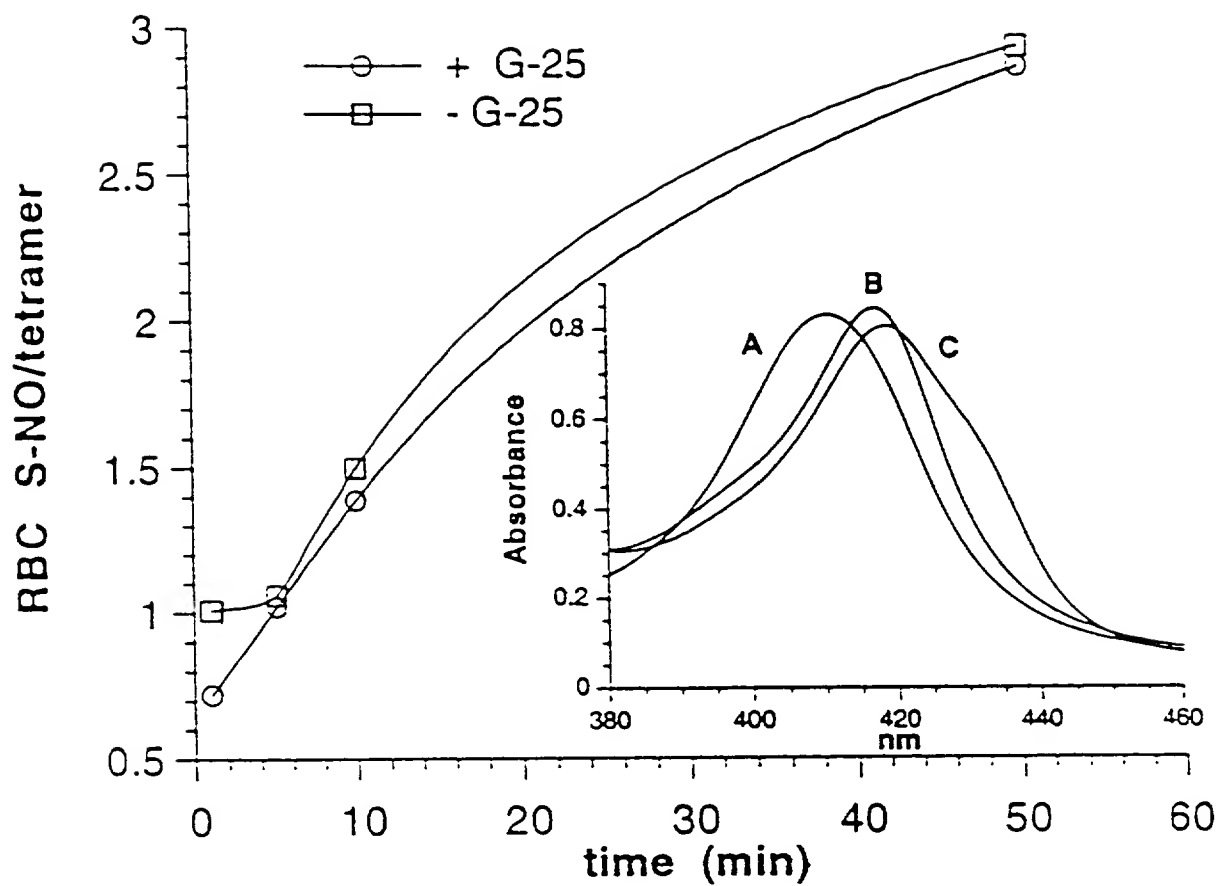


FIG. 3A

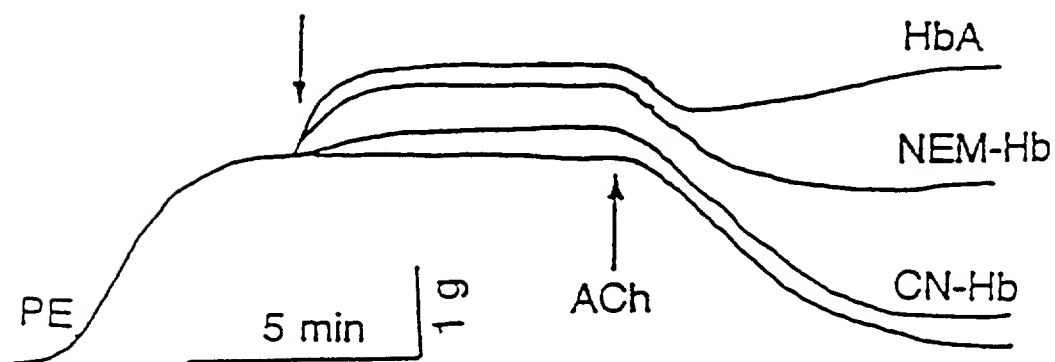


FIG. 3B

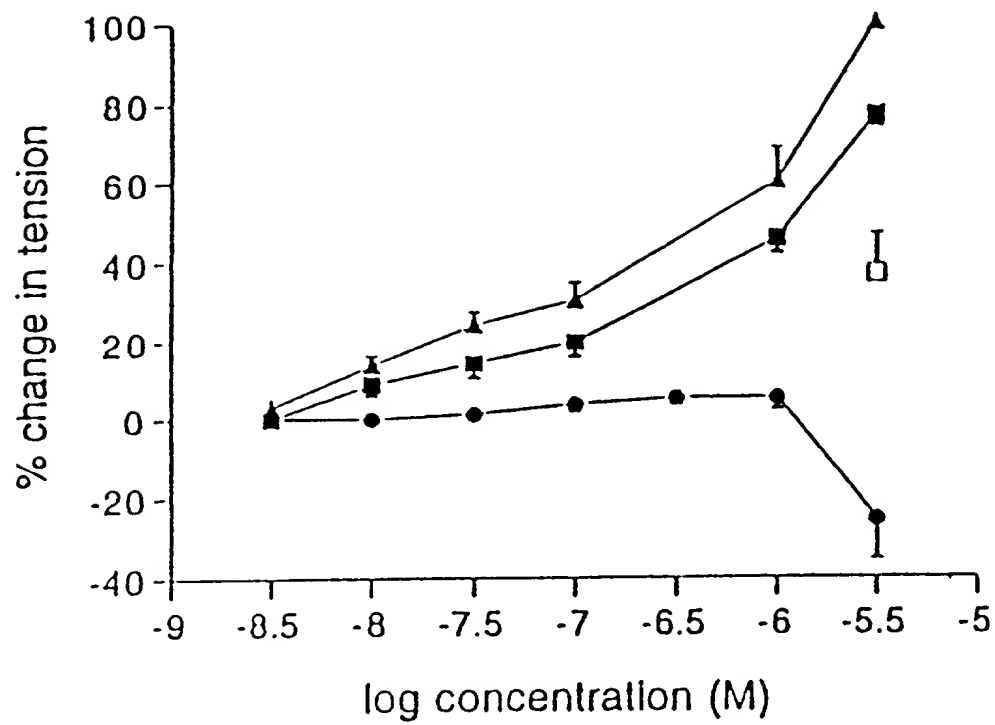


FIG. 4A

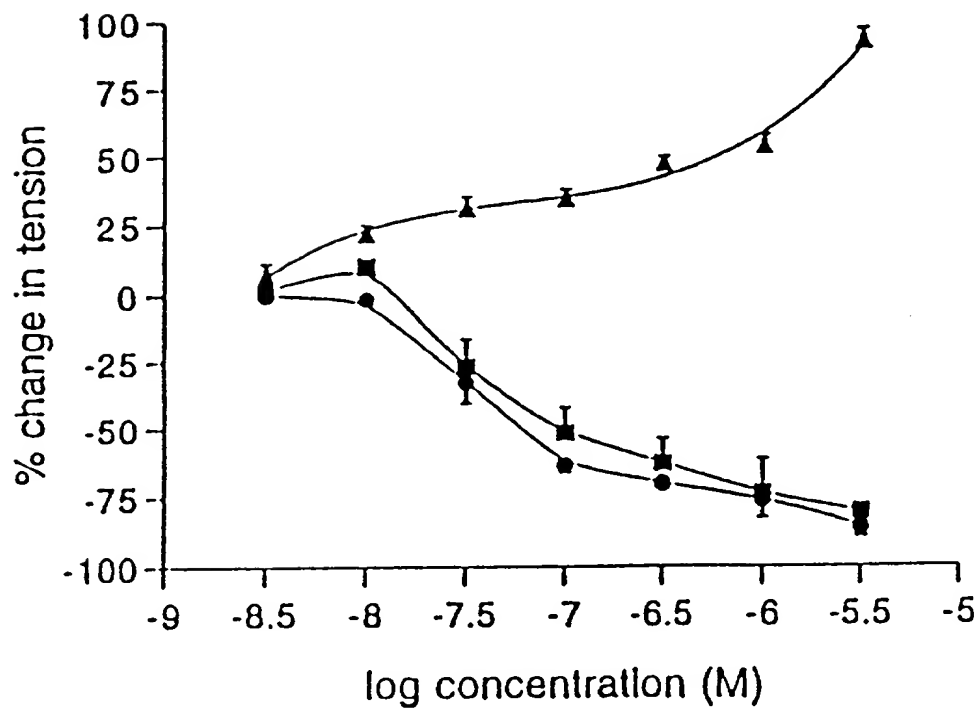


FIG. 4B

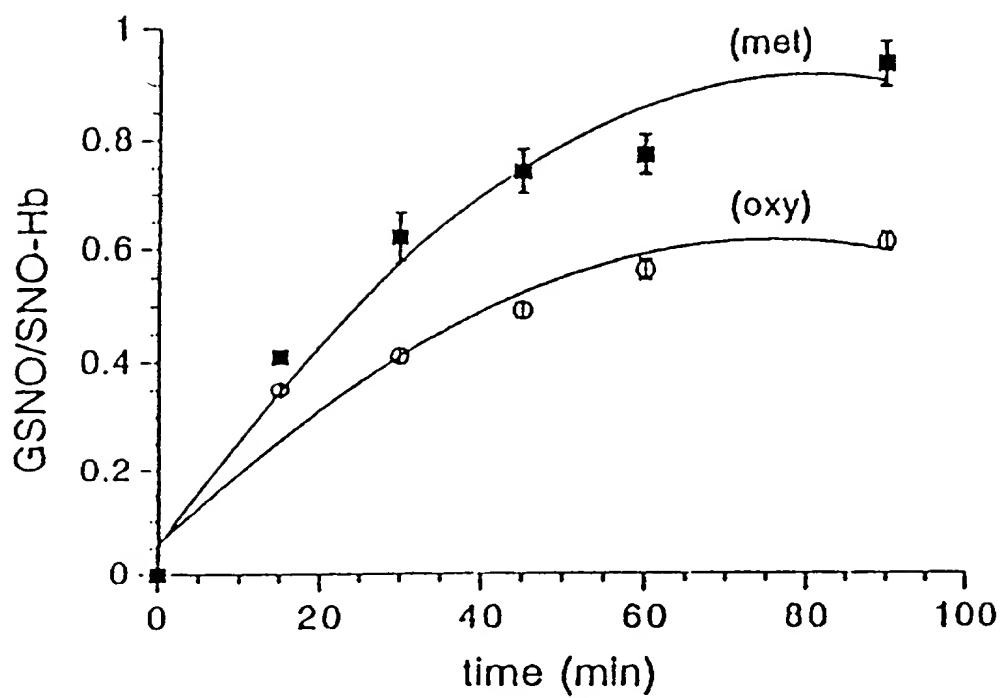


FIG. 4C

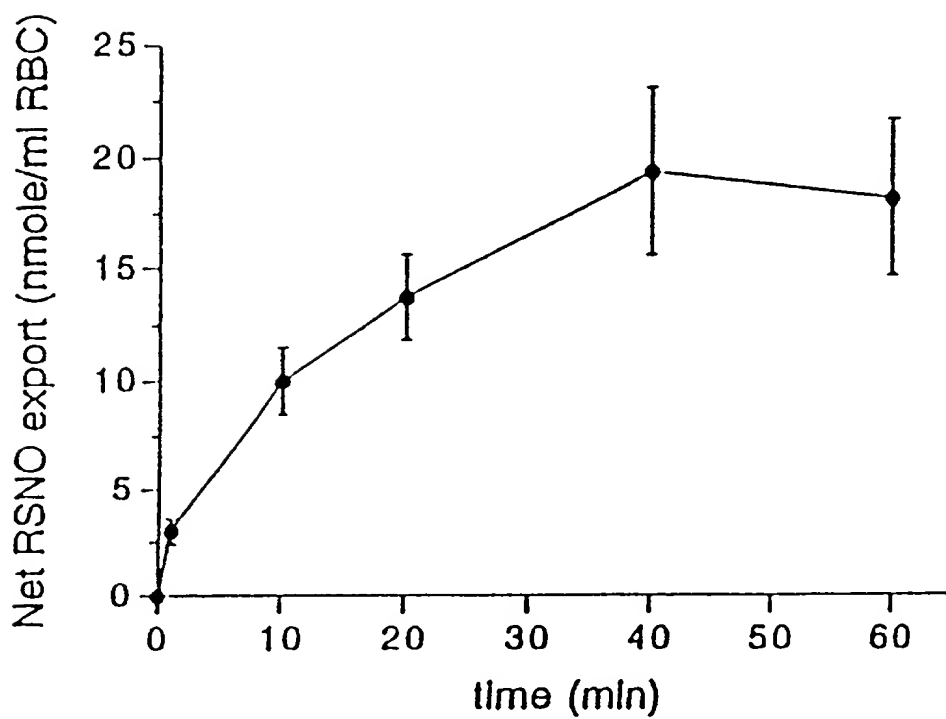


FIG. 4D

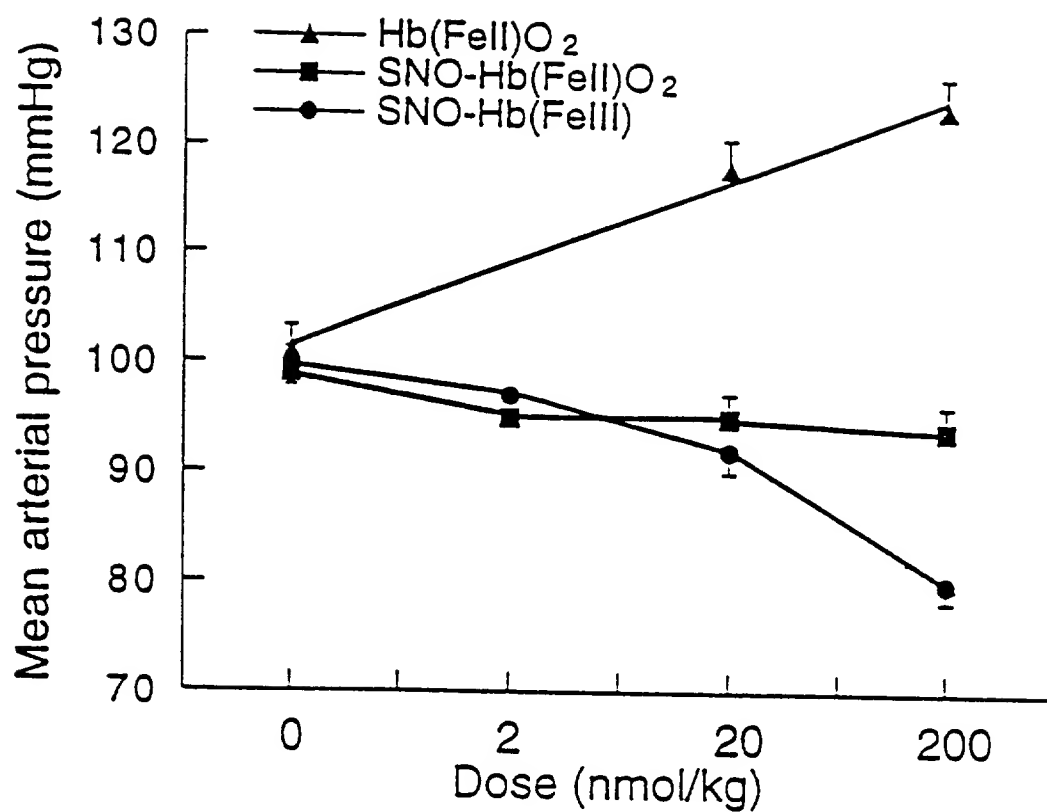
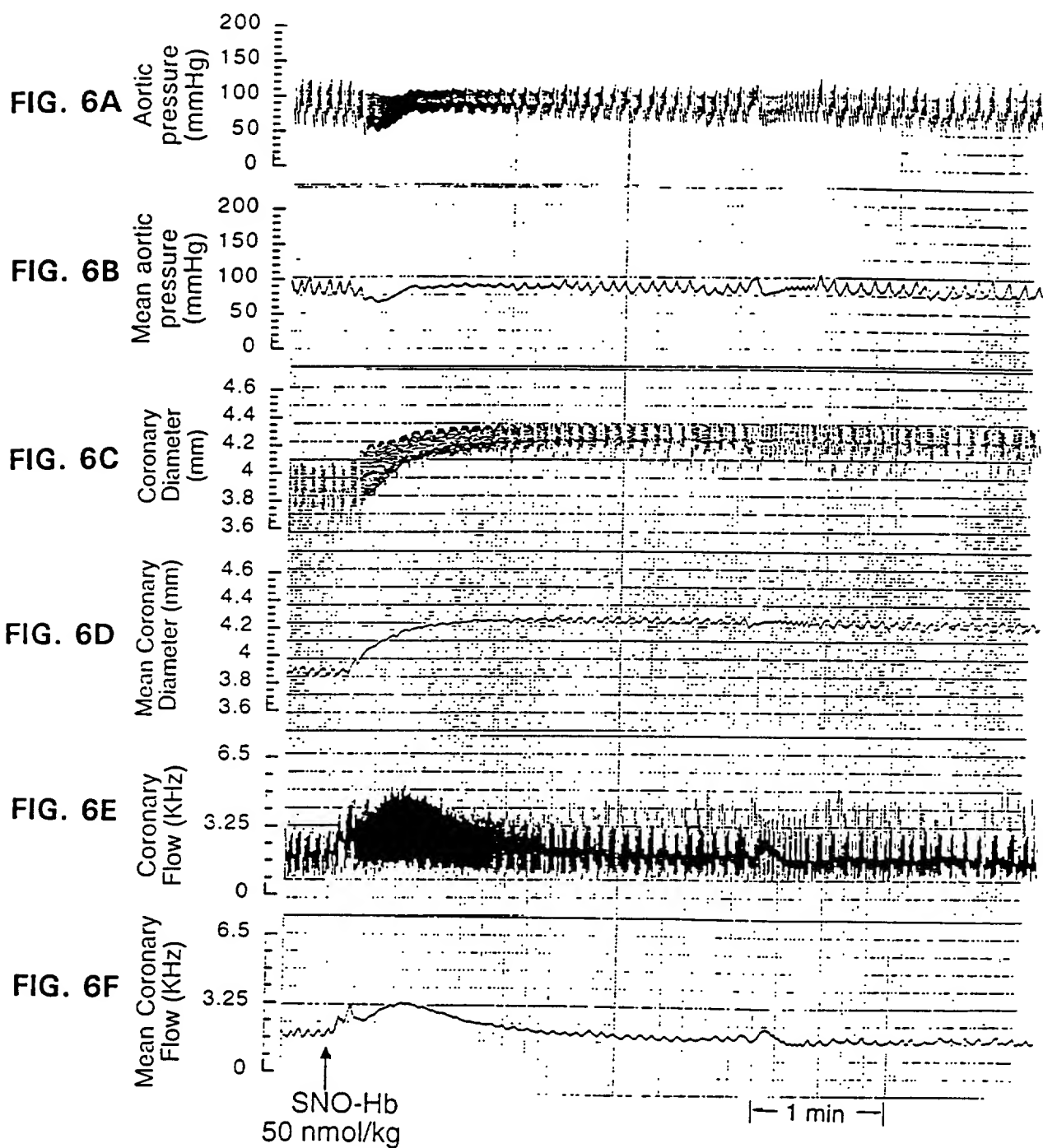


FIG. 5



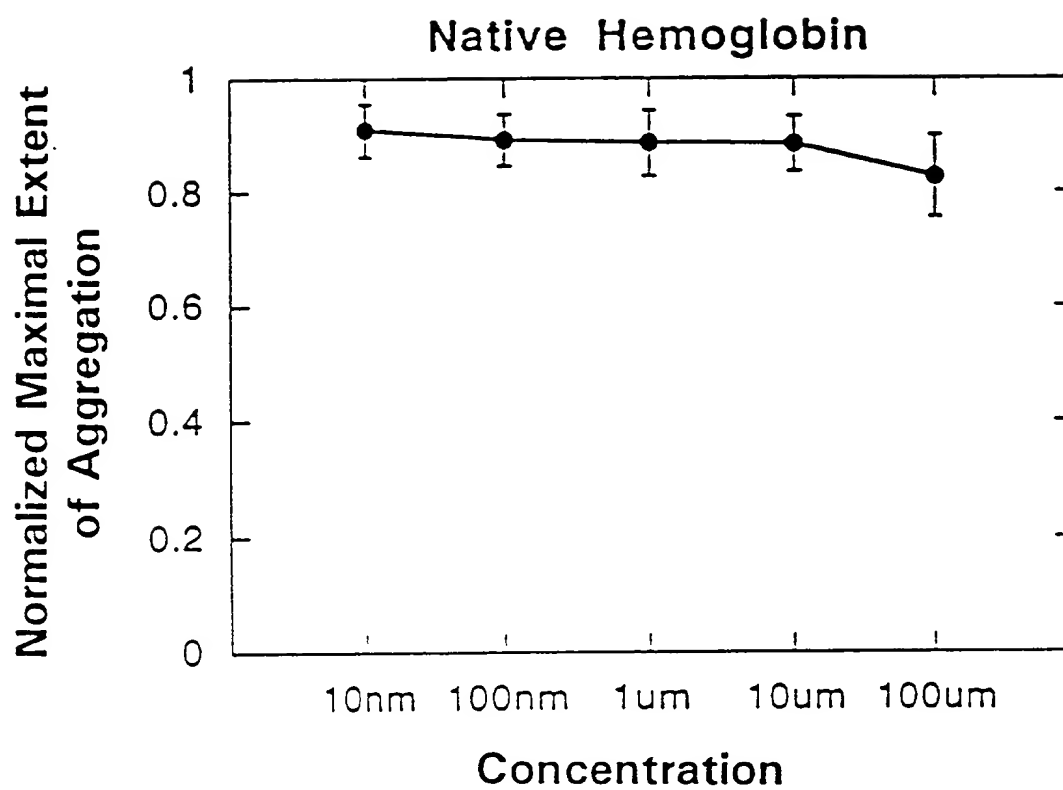


FIG. 7A

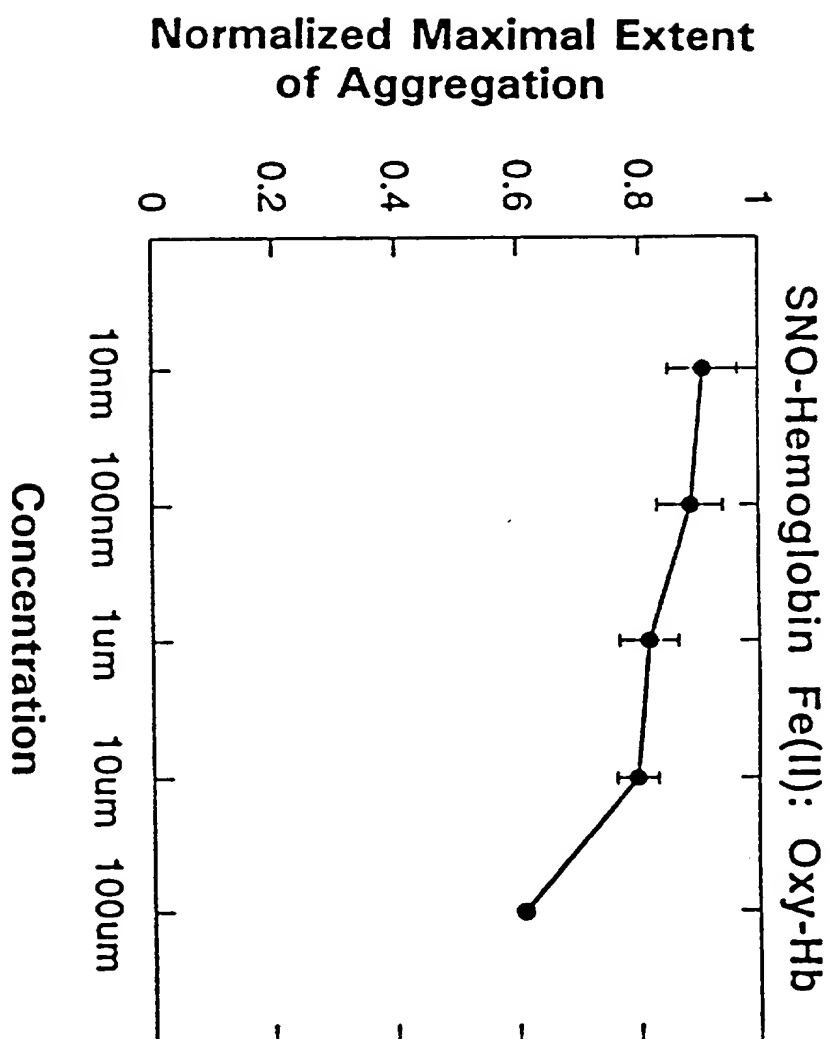


FIG. 7B

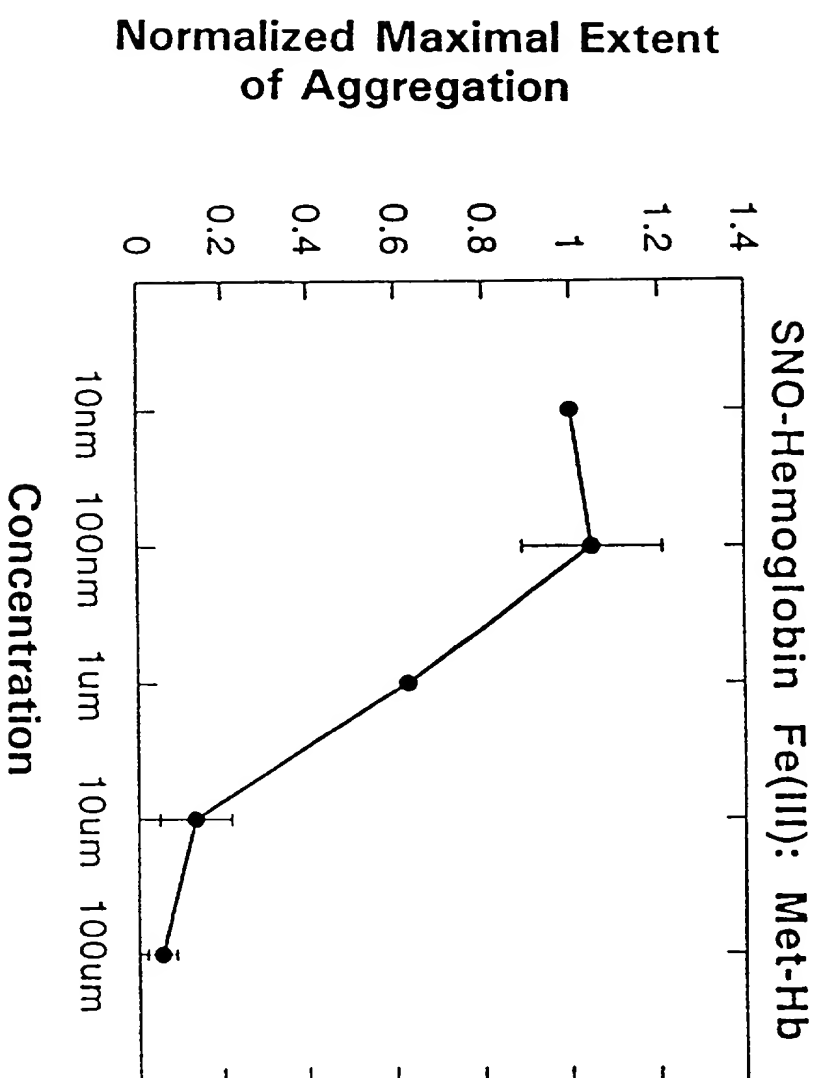


FIG. 7C

(cGMP) Under the Effect of Various Types of Hemoglobin

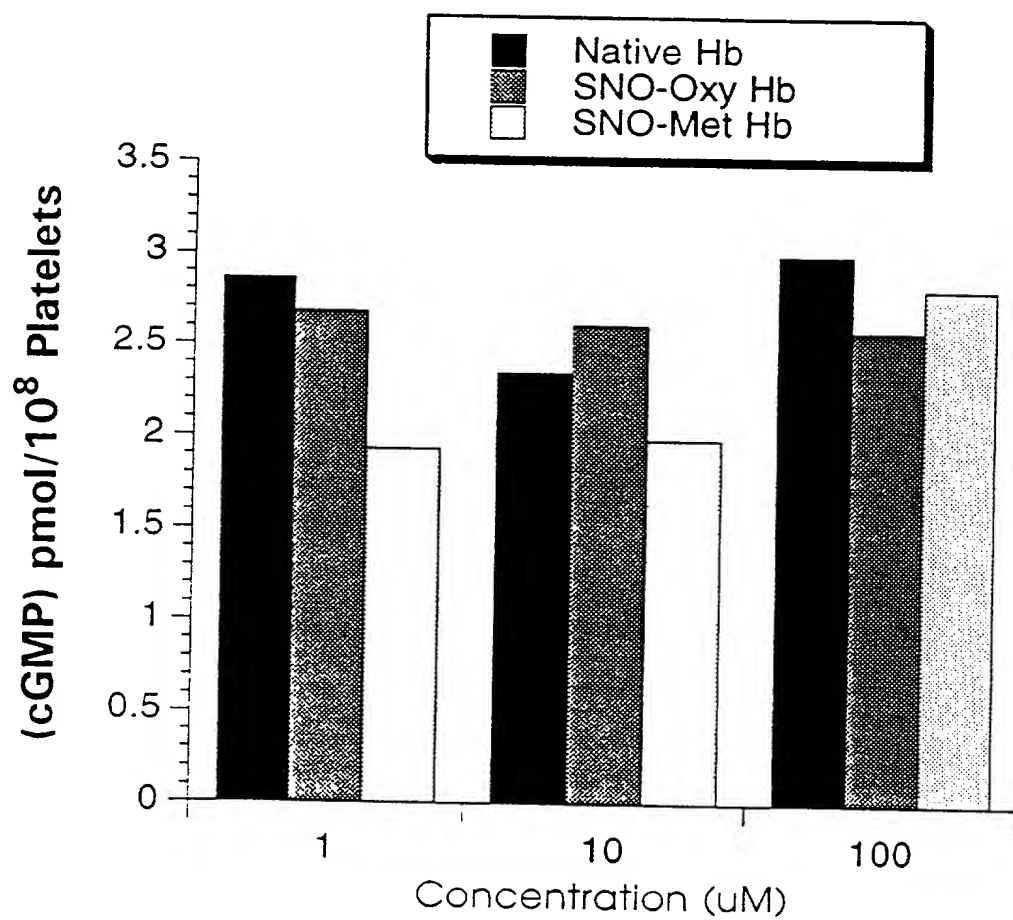


FIG. 8

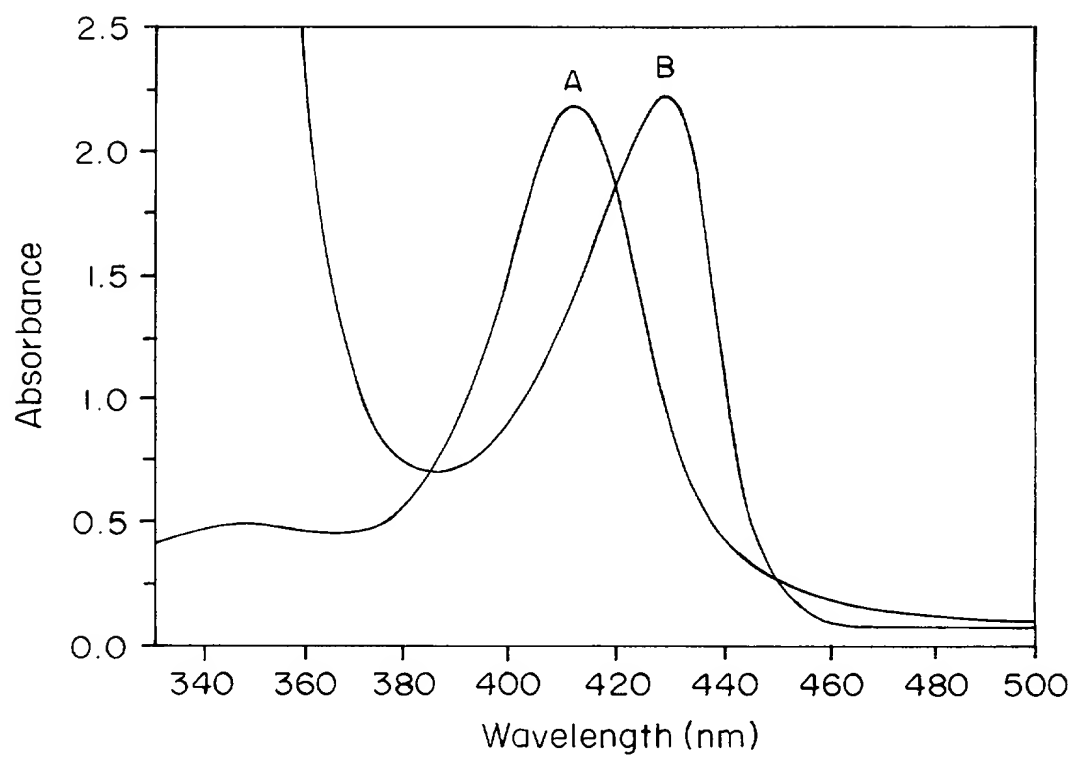


FIG. 9A

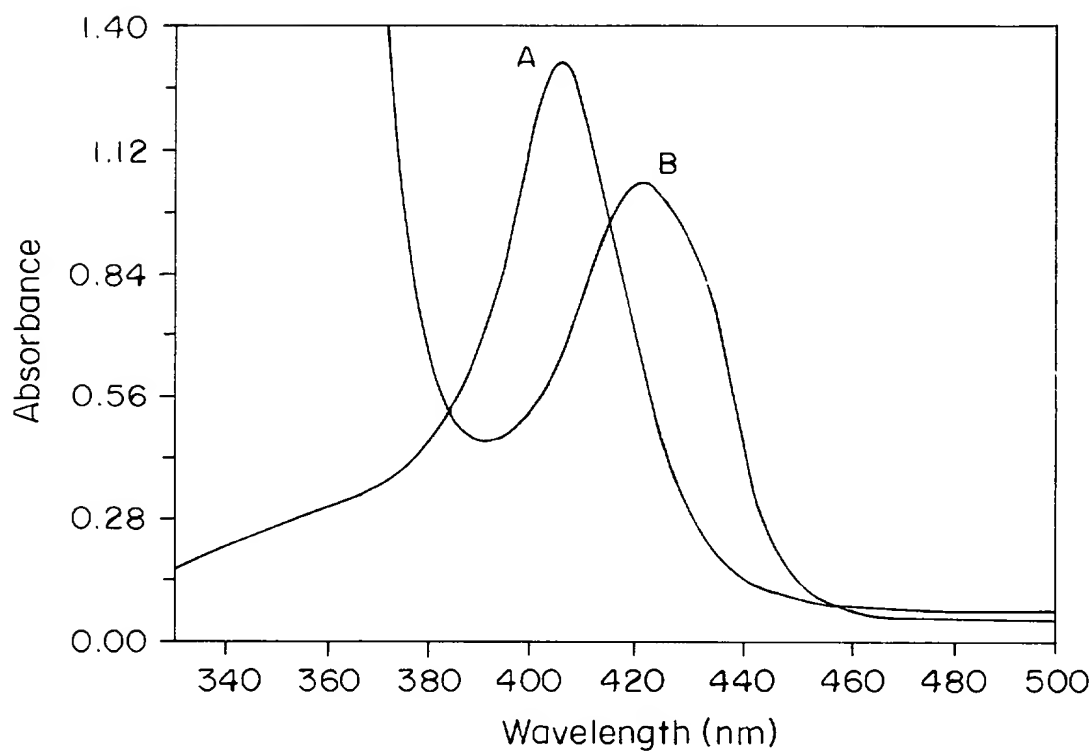


FIG. 9B

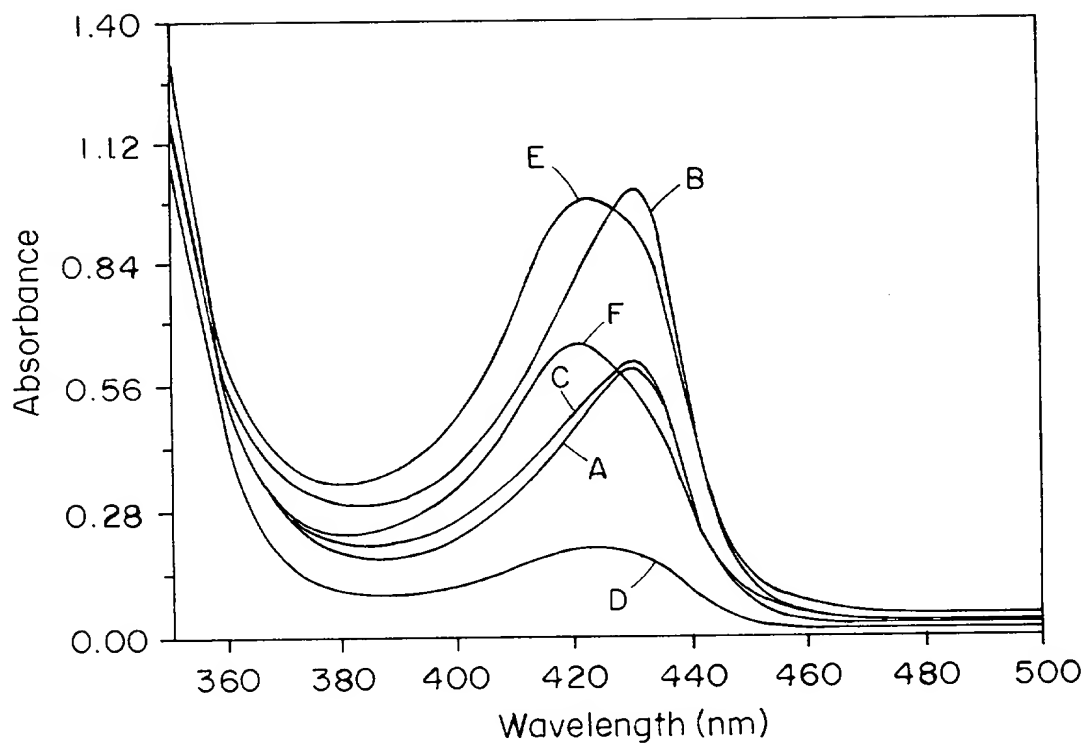


FIG. 9C

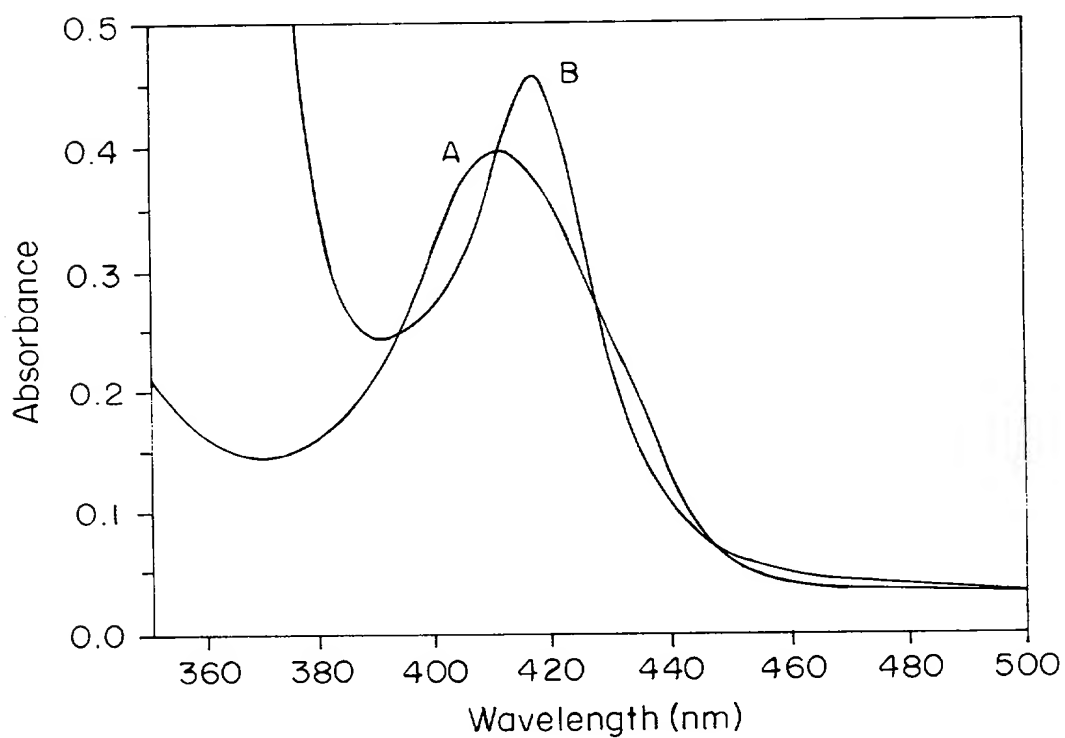


FIG. 9D

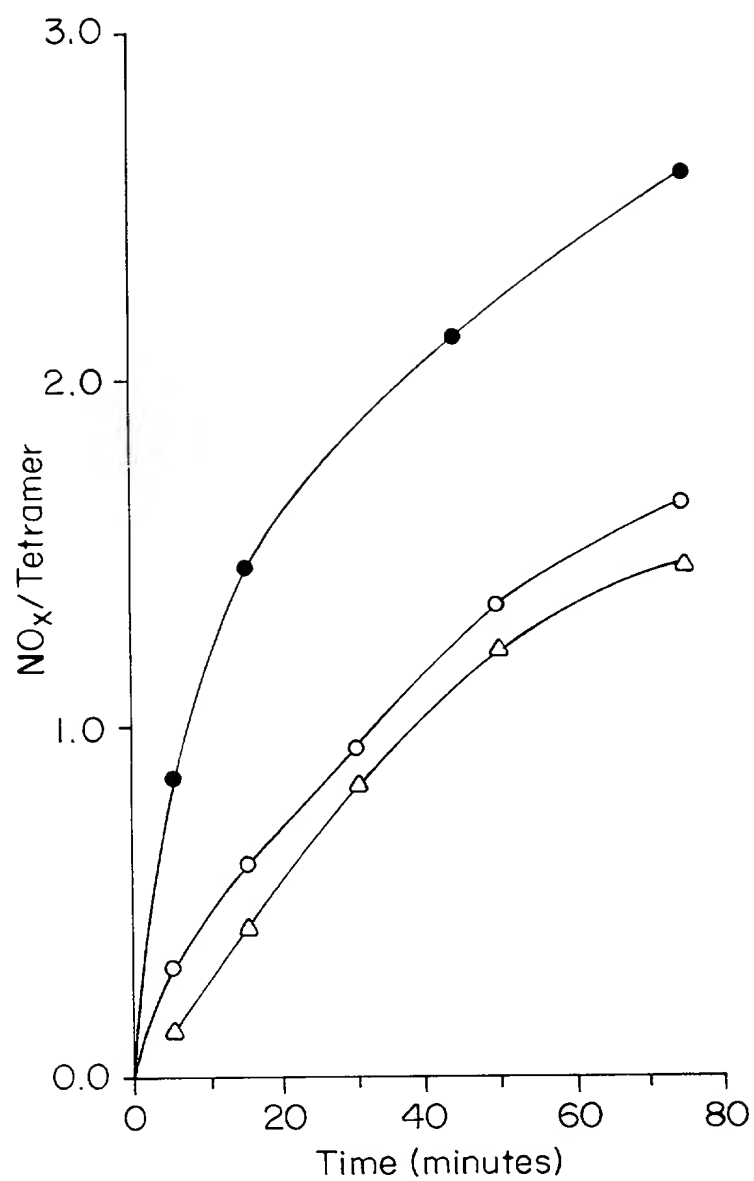


FIG. 9E

Change in Blood Flow in Rat Caudatoputamen Nucleus
after Injecting SNO-Hb to Rats Breathing in 21% O₂

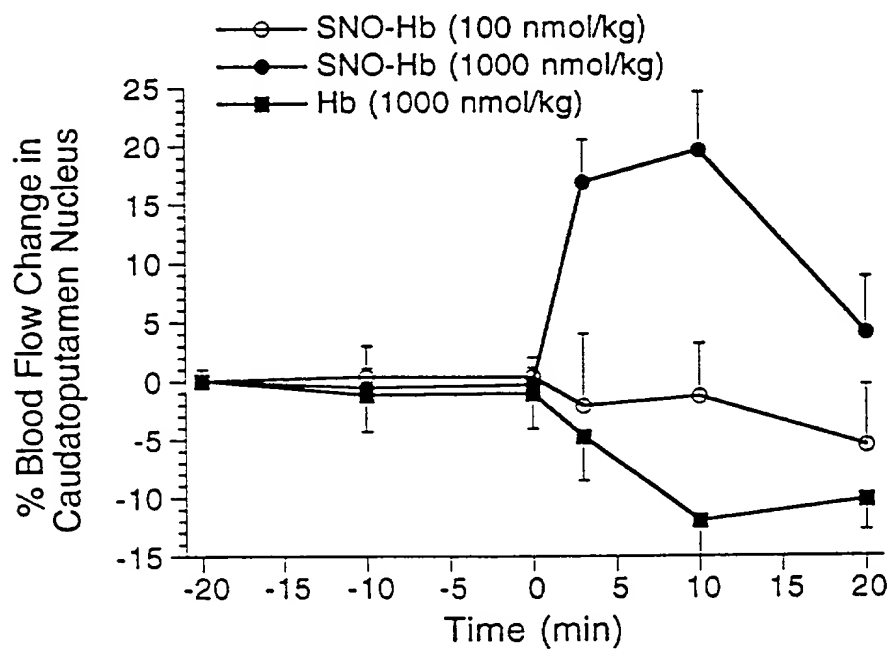


FIG. 10

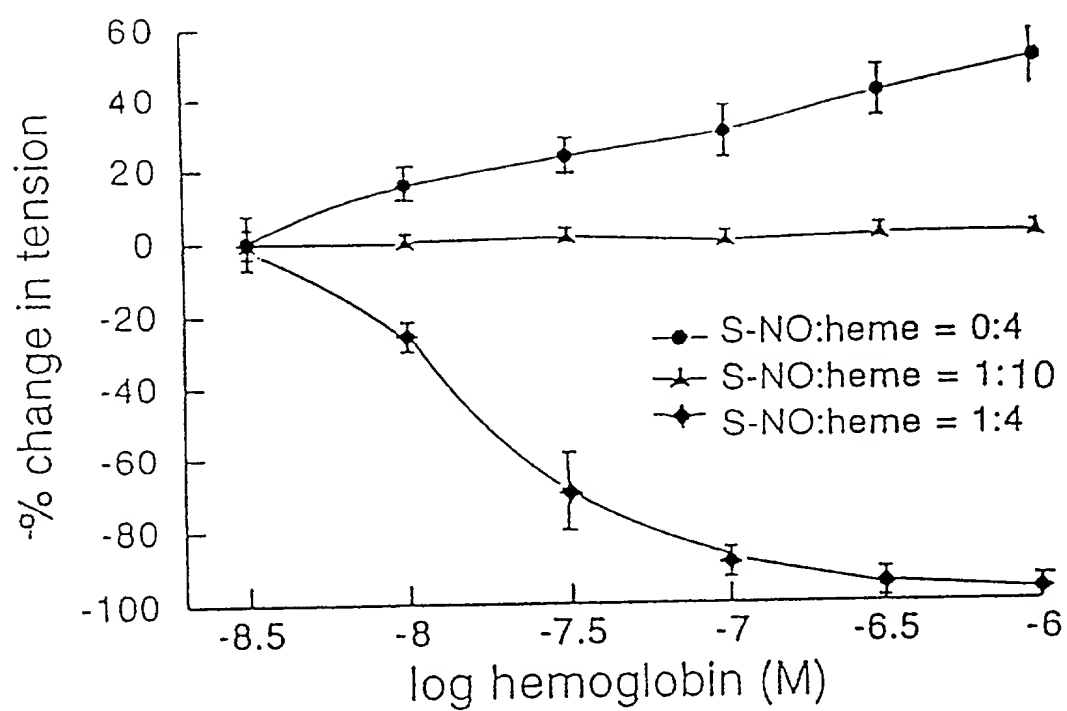
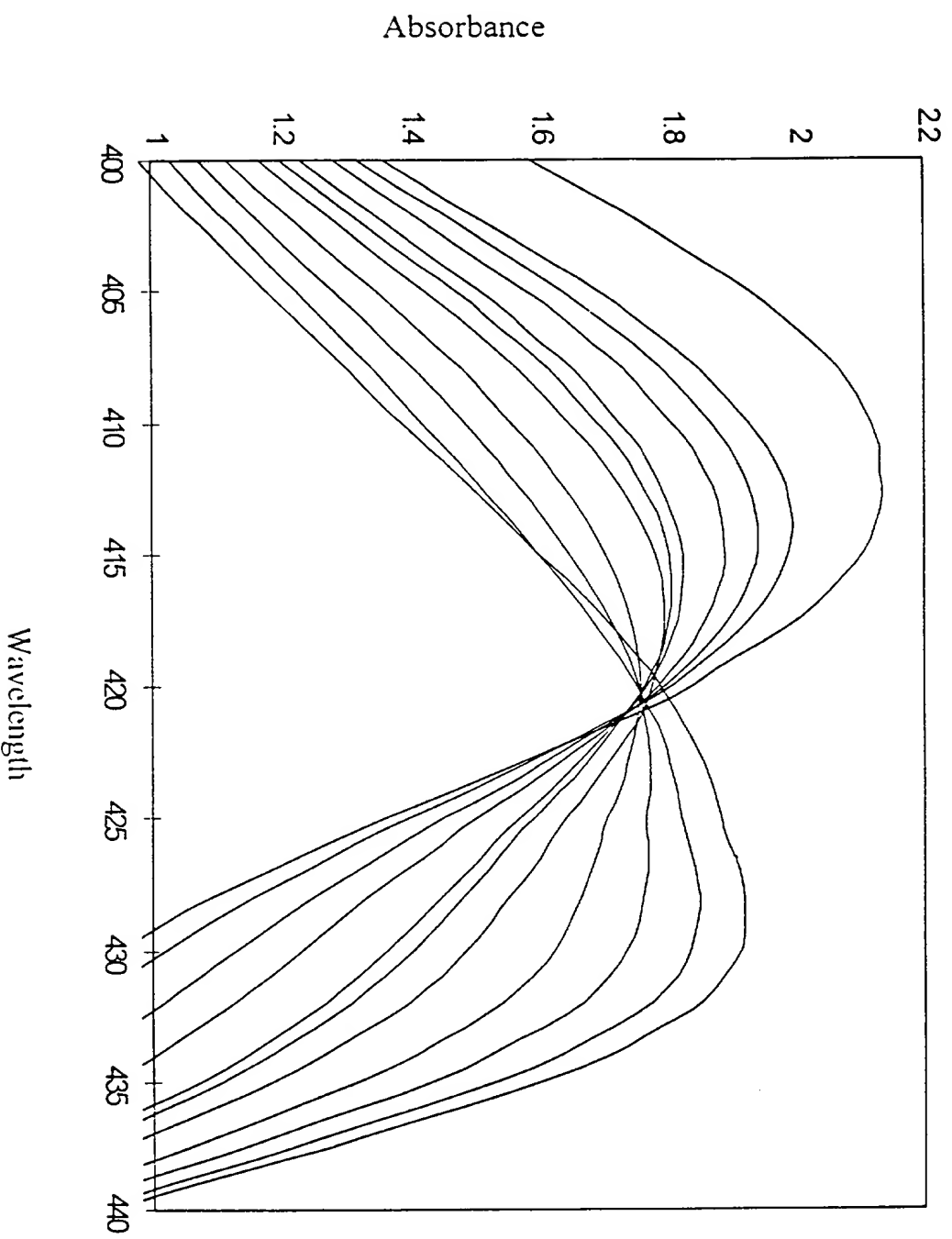


FIG. 11

FIG. 12



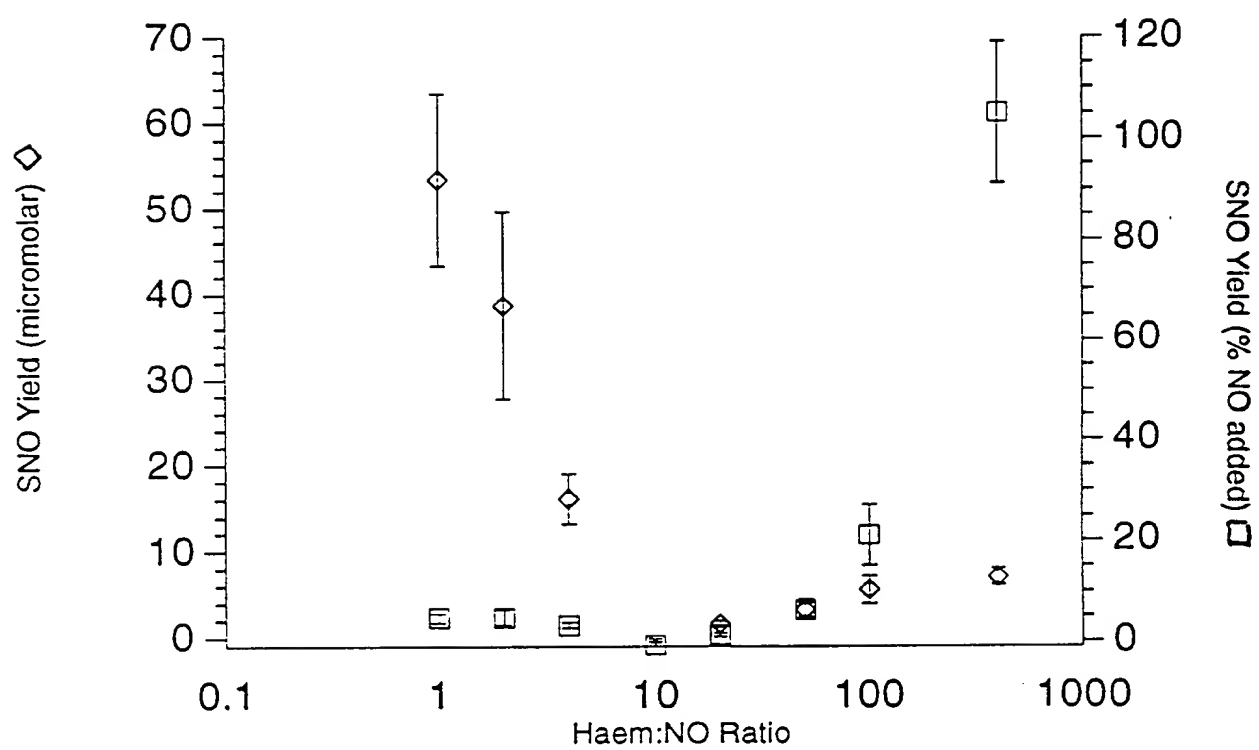


FIG. 13

FIG. 14A

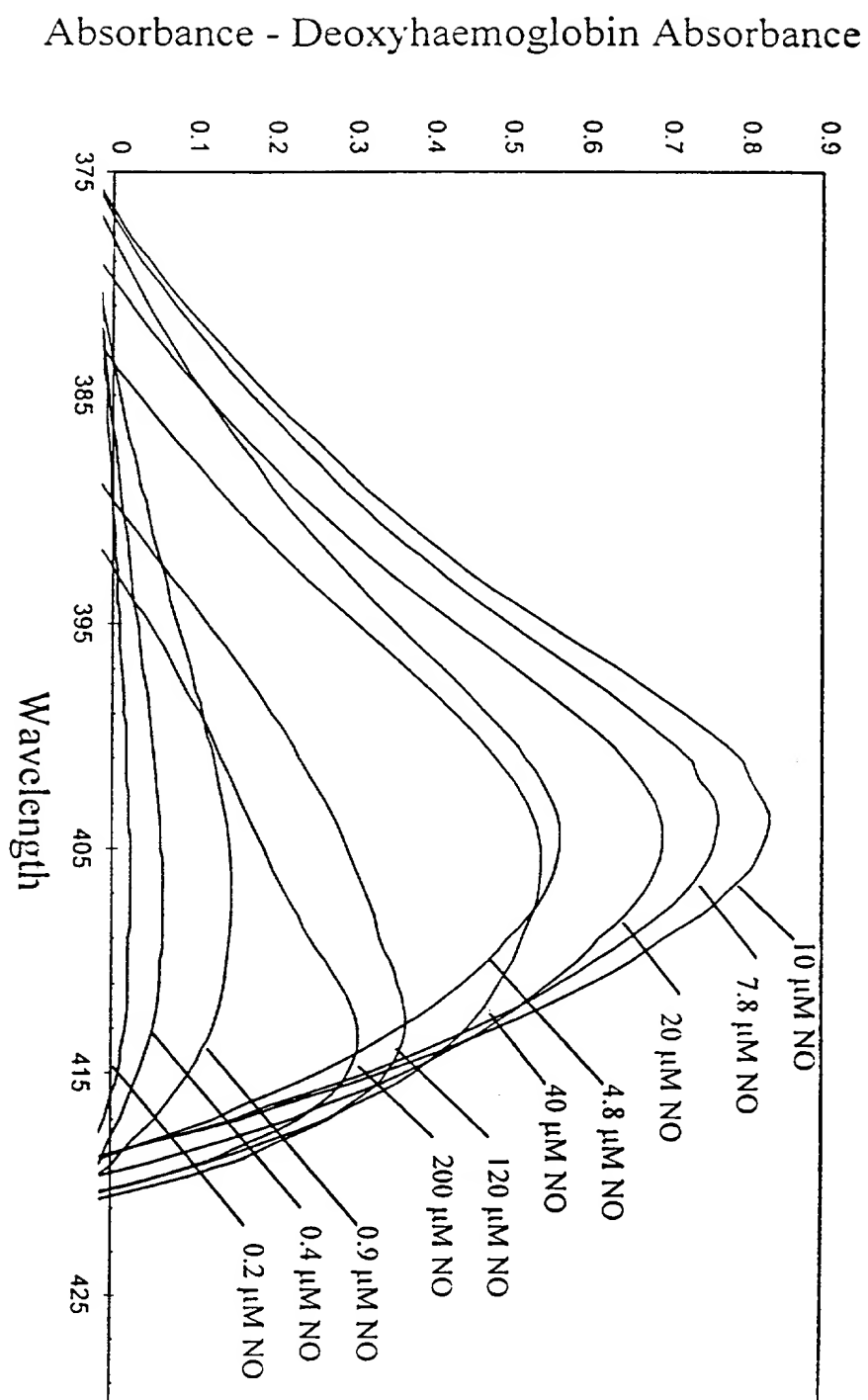


FIG. 14B

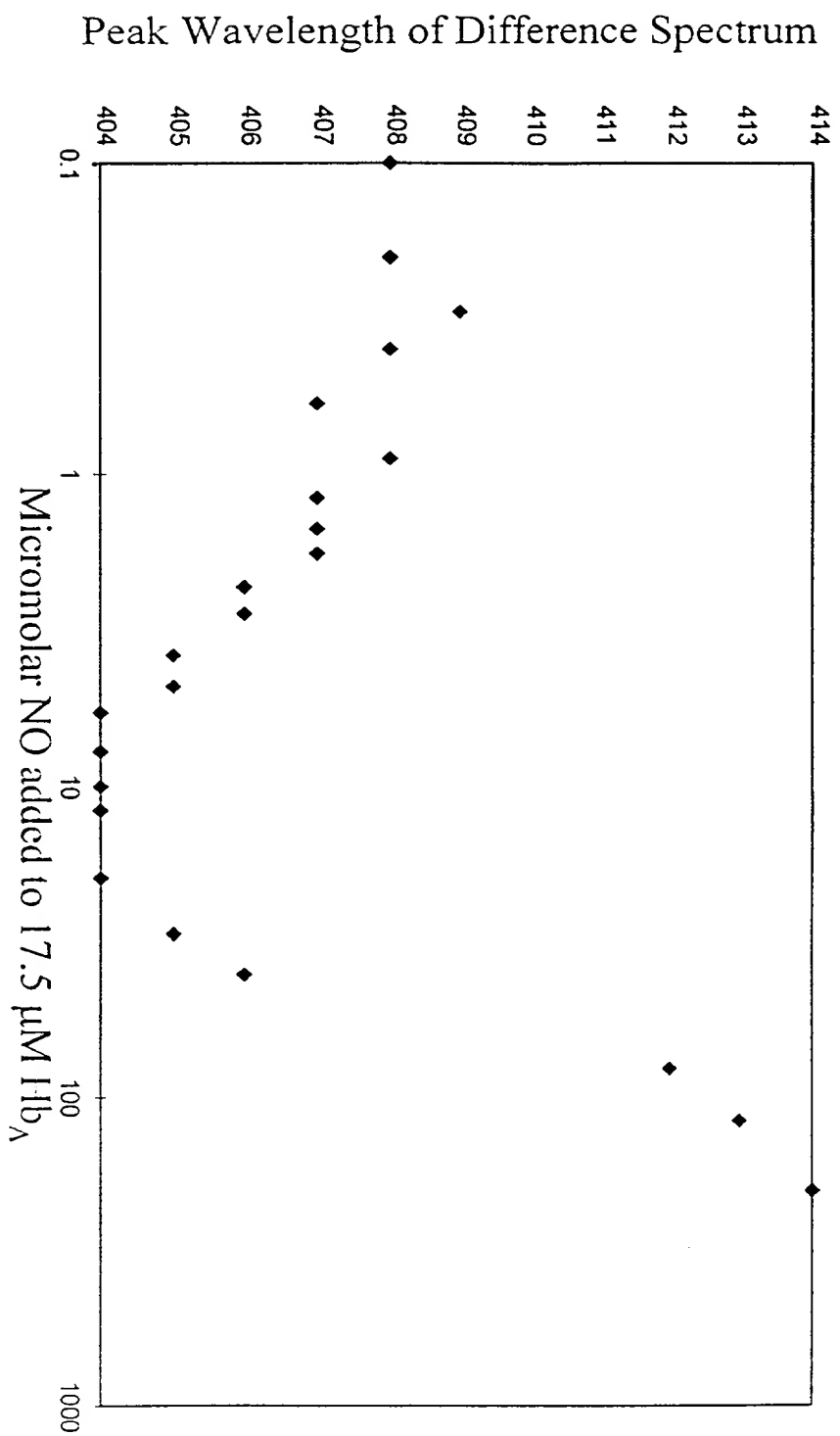


FIG. 15A

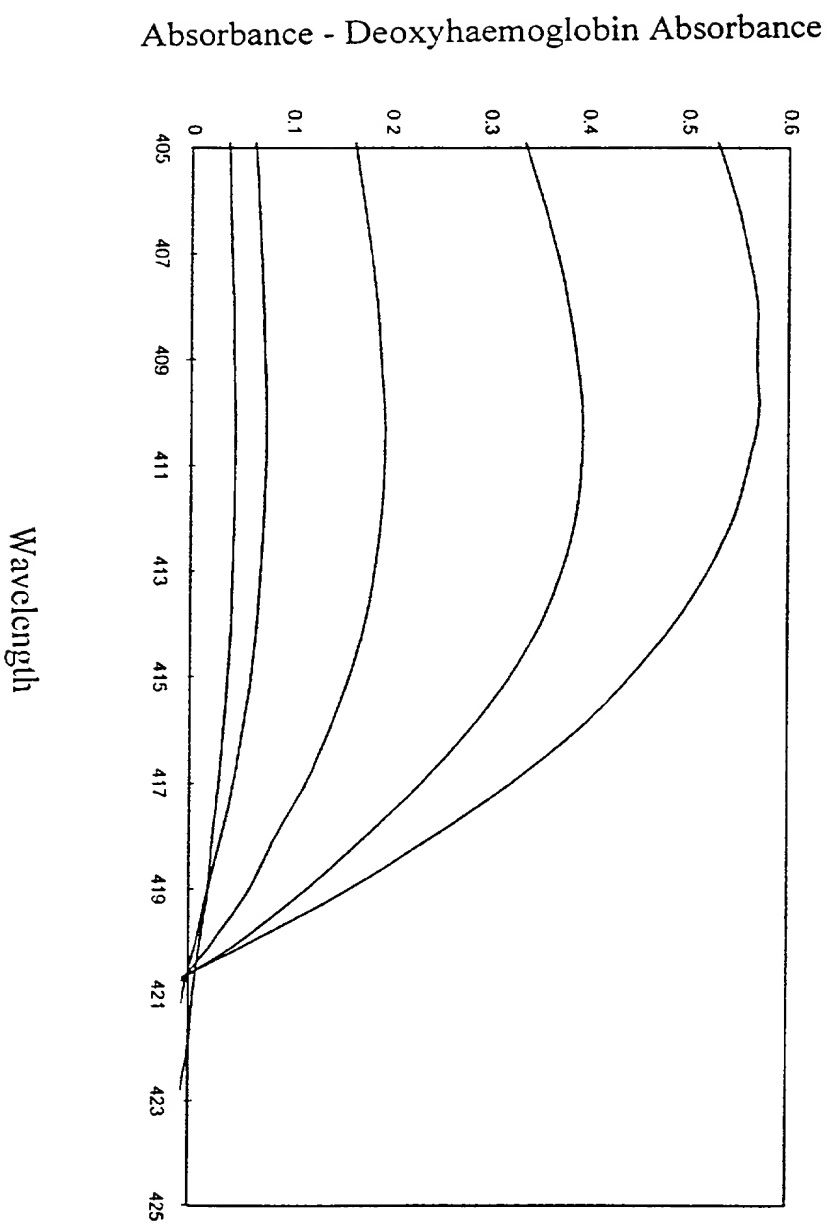


FIG. 15B

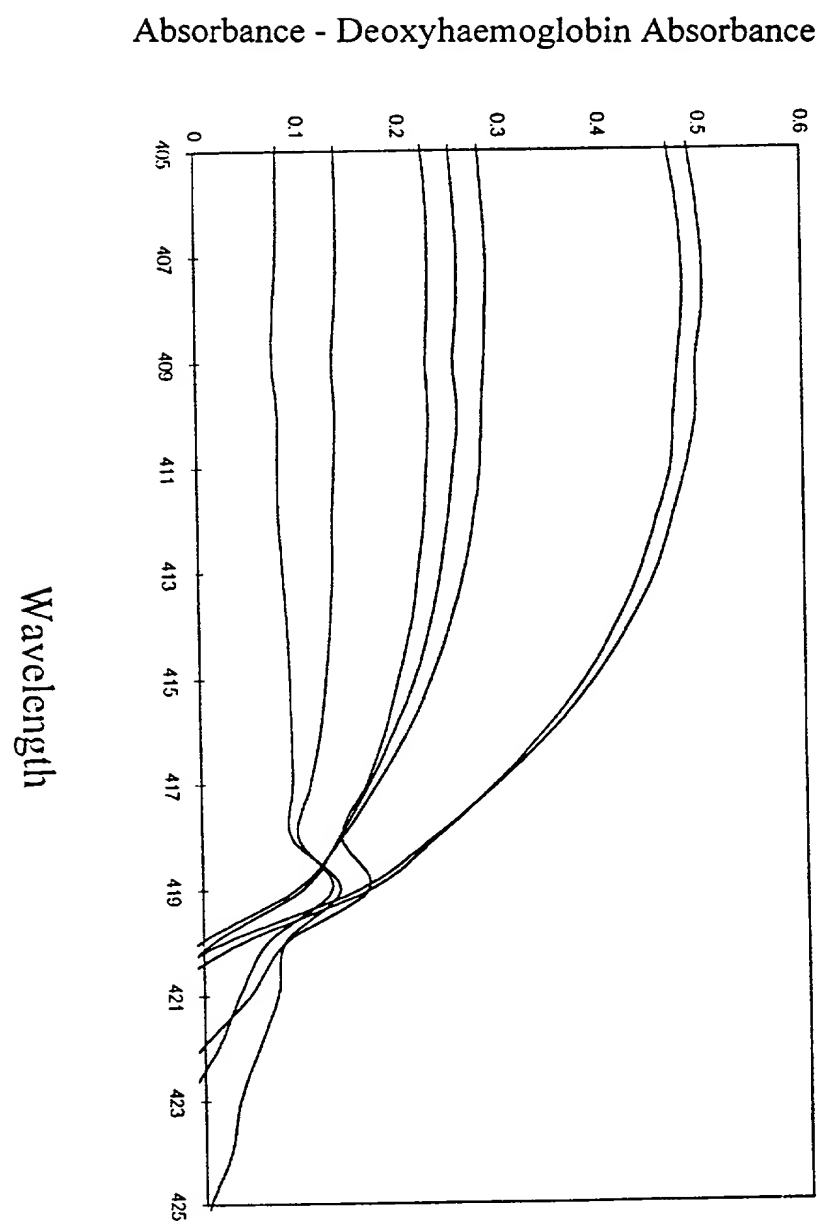
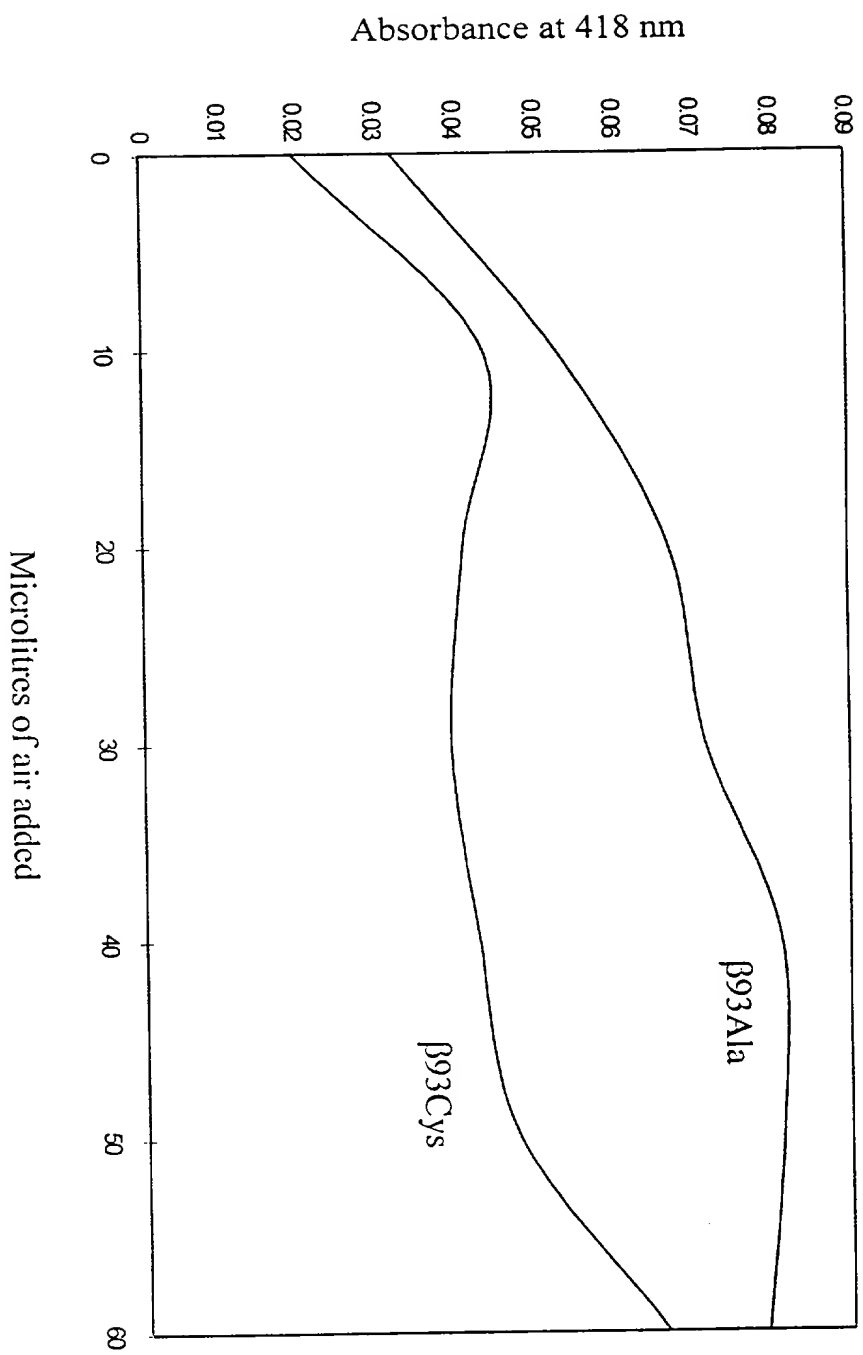


FIG. 16



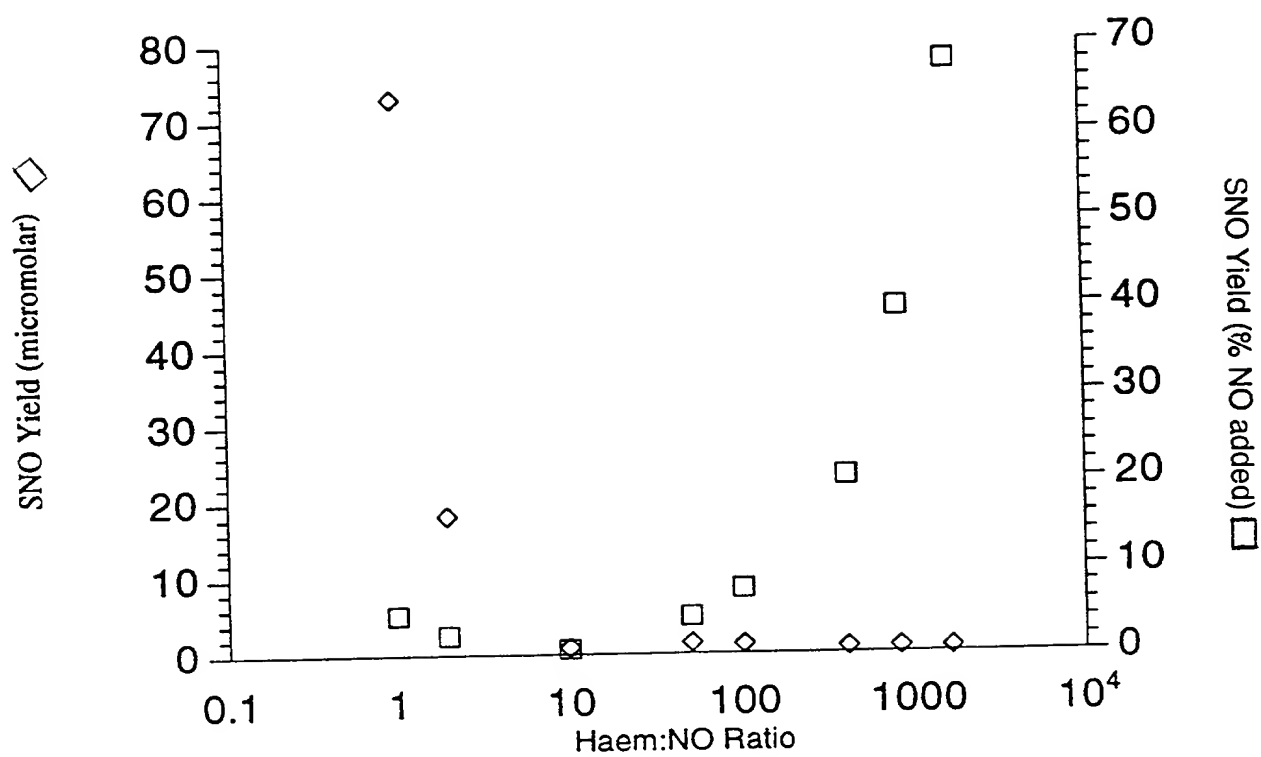


FIG. 17

FIG. 18A

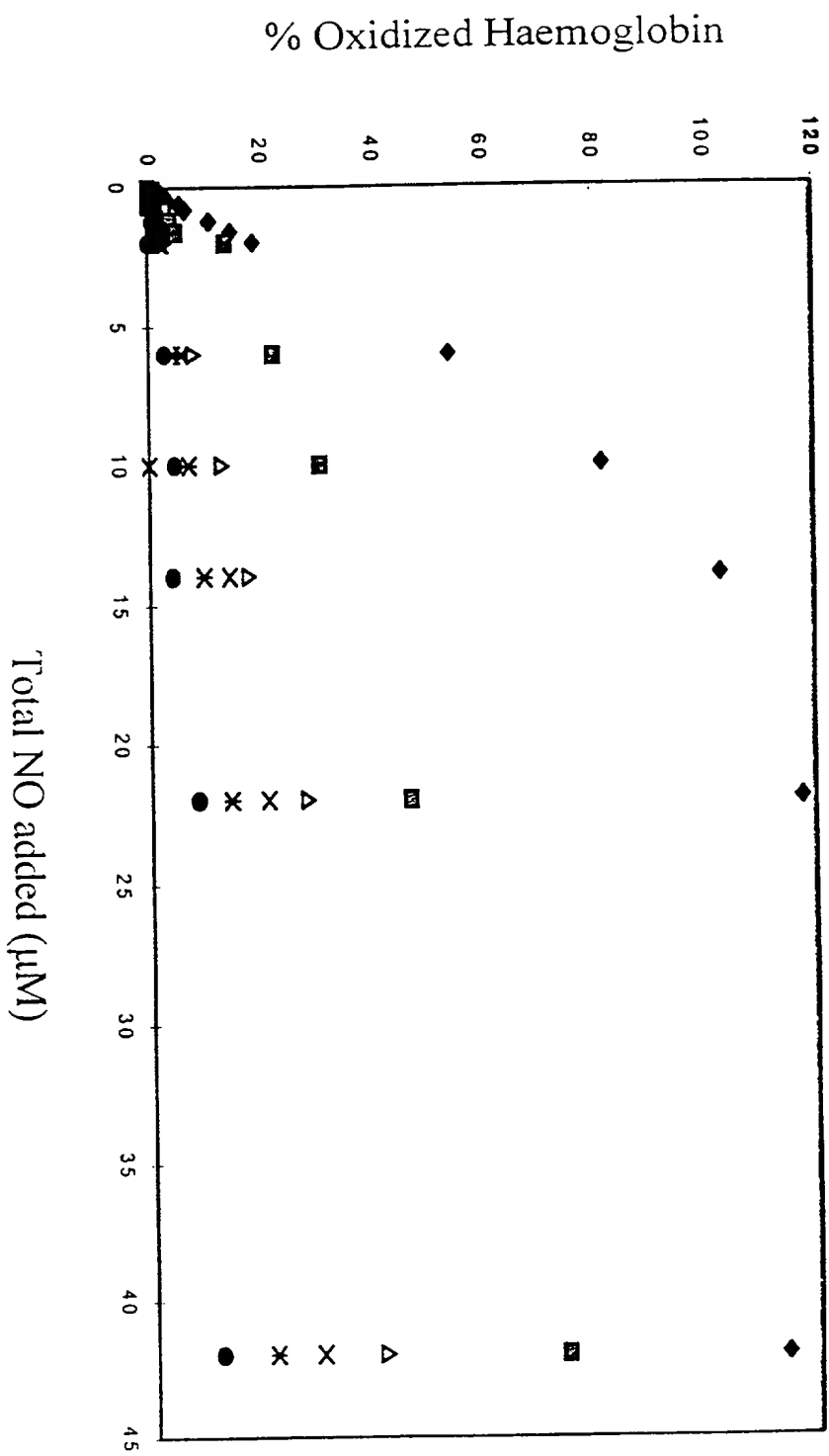
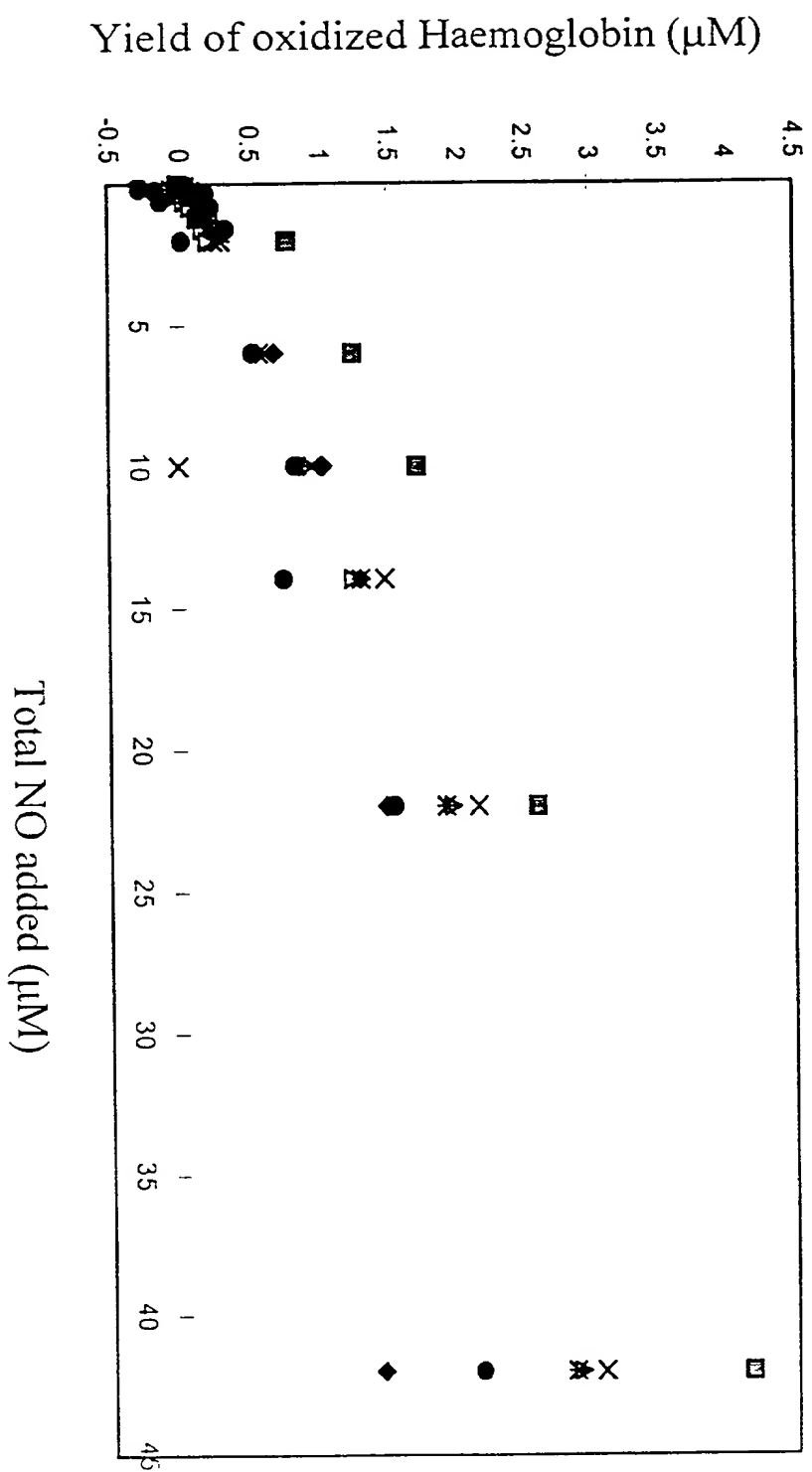
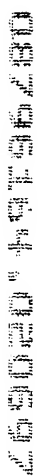


FIG. 18B



— — — — —



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Declaration for Patent Application

As a named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name;

I believe I am the original, first and sole inventor (if only one name is listed) or an original, first and joint inventor (if plural names are listed in the signatory page(s) commencing at page 3 hereof) of the subject matter which is claimed and for which a patent is sought on the invention entitled

MODIFIED HEMOGLOBINS, INCLUDING NITROSYLHEMOGLOBINS, AND USES THEREFOR

the specification of which (check one)

☒ is attached hereto.

☐ was filed on _____ as United States Application Number or PCT International Application Serial No. _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

<u>Prior Foreign Application(s)</u>			Priority Not Claim
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[]
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[]
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[]

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

60/003,801
(Application Number)

September 15, 1995
(Filing Date)

(Application Number)

(Filing Date)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information known by me to be material to patentability as defined in 37 C.F.R. §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

<u>PCT/US96/14659</u>	<u>September 13, 1996</u>	<u>Pending</u>
(Application Serial No.)	(Filing date)	(Status, patented, pending, abandoned)

<u>08/667,003</u>	<u>June 20, 1996</u>	<u>Pending</u>
(Application Serial No.)	(Filing date)	(Status, patented, pending, abandoned)

<u>08/616,371</u>	<u>March 15, 1996</u>	<u>Pending</u>
(Application Serial No.)	(Filing date)	(Status, patented, pending, abandoned)

_____	_____	_____
(Application Serial No.)	(Filing date)	(Status, patented, pending, abandoned)

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

I also hereby grant additional Powers of Attorney to the following attorney(s) and/or agent(s) to file and prosecute an international application under the Patent Cooperation Treaty based upon the above-identified application, including a power to meet all designated office requirements for designated states:

David E. Brook	Reg. No. 22,592	Thomas O. Hoover	Reg. No. 32,470
James M. Smith	Reg. No. 28,043	Alice O. Carroll	Reg. No. 33,542
Leo R. Reynolds	Reg. No. 20,884	N. Scott Pierce	Reg. No. 34,900
Patricia Granahan	Reg. No. 32,227	Richard A. Wise	Reg. No. 18,041
Mary Lou Wakimura	Reg. No. 31,804	Carol A. Egner	Reg. No. 38,866

all of Hamilton, Brook, Smith and Reynolds, P.C., Two Militia Drive, Lexington, Massachusetts 02173;

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(617) 861-6270

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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